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(54) Title: PREVENTION AND TREATMENT OF AGING AND NEURODEGENERATIVE DISEASES

(57) Abstract: This application provides methods of identifying agents to treat or prevent neurodegenerative disease, and methods of using such agents, such as those that reduce mitochondrial ATP synthase (ATPsyn) activity resulting in the modulation of 5' AMP-activated protein kinase (AMPK) activity, ATP production, NAD⁺ production, NADH production, NADPH production and/or mitochondrial calcium levels, to treat or prevent a neurodegenerative disease. Also provided are compounds and methods of their use to treat or prevent (e.g., delay one or more signs of) aging. In some examples, decreasing mTor activity can be used to treat aging.



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PREVENTION AND TREATMENT OF AGING AND NEURODEGENERATIVE DISEASES

CROSS-REFERNCE TO RELATED APPLICATIONS

5 This application claims priority to U.S. Provisional Application Nos. 62/196,211 filed July 23, 2015, and 62/196,143 filed July 23, 2015, both herein incorporated by reference.

FIELD

10 Provided are methods of identifying agents to treat or prevent neurodegenerative disease, and methods of using such agents, such as those that reduce mitochondrial ATP synthase (ATPsyn) activity resulting in the modulation of 5' AMP-activated protein kinase (AMPK) activity, ATP production, NAD⁺ production, NADH production, NADPH production and/or mitochondrial calcium levels, to treat or prevent a neurodegenerative disease. The disclosure also provides methods of using J147 and related compounds to treat or prevent (*e.g.*, delay one or more signs of)
15 aging.

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BACKGROUND

25 There is currently no drug to prevent or slow down the progression of Alzheimer's disease (AD) pathology, perhaps due, at least in part, to the use of inappropriate *in vitro* and *in vivo* disease models for drug screening. J147, a synthetic derivative of the curry spice curcumin, is a potent neuroprotective small molecule that is orally active in memory and transgenic AD animal models [1, 2, 3]. Not only is J147 superior to the most commonly used AD drug, Donepezil, at enhancing memory in normal rodents, it also prevents cognitive decline in APP/swePS1ΔE9 mice [2, 3]. J147 reverses the cognitive deficits in APP/swePS1ΔE9 mice when administered at very late stages in
30 the disease [3].

 Although AD transgenic rodents have been used as state of the art models for AD drug candidate testing, the vast majority rely on familial AD (FAD) mutations. Unfortunately, none of the drug candidates identified using these models has translated to the clinic. Given that FAD

accounts for less than 1% of all AD cases [4], animal models that more accurately reflect the predominant sporadic forms of AD and other dementias are needed [5].

Age is by far the greatest risk factor for dementia. One model of aging is the senescence-accelerated prone 8 (SAMP8) mouse, that has a progressive, age-associated decline in brain function similar to human AD patients (reviewed in [6, 7, 8]). As they age, SAMP8 mice develop an early deterioration in learning and memory as well as a number of pathophysiological alterations in the brain including increased oxidative stress, inflammation, vascular impairment, gliosis, A β accumulation and tau hyperphosphorylation. Therefore, the SAMP8 mice together with their response to J147 may help to delineate an understanding of the molecular mechanisms that are shared by aging and disease. These insights could lead to novel interventions for old age-associated sporadic AD.

To investigate the interaction between aging and the AD drug candidate J147 on brain function as well as brain and systemic metabolism, an integrative multi-omics approach was carried out in SAMP8 mice. Changes in behavior, protein expression, levels of metabolites and the whole transcriptome in old SAMP8 mice fed with control or J147 diets were compared with young SAMP8 control mice. These data identify a subset of metabolic changes associated with aging that may be relevant to sporadic AD and other forms of dementia. The data demonstrate the ability of J147 to suppress many of these changes, while reducing some aspects of aging.

mTOR is a central regulator of many aspects of cell physiology (Laplane and Sabatini, J. Cell Sci. 122, 3589-3594, 2009). Its inhibition by rapamycin extends lifespan in mice (Laplane and Sabatini, J. Cell Sci. 122, 3589-3594, 2009). mTORC1 positively regulates protein synthesis via the phosphorylation of eukaryotic initiation factor 4E (eIF4E) binding protein (4E-BP1) and p70 S6 Kinase 1 (S6K1). Under conditions of stress, mTORC1 is inhibited and the activities of S6K1 and 4E-BP1 are reduced. In contrast, mTORC1 represses autophagy by phosphorylating ULK-1; inhibiting mTOR increases autophagy that can be neuroprotective. mTORC1 is central to the integration of many signals. One is tuberous sclerosis complexes 1 and 2, which are responsive to energy, and oxygen levels as well as growth factor stimulation. AMPK is also responsive to AMP/ADP levels, and can reduce mTORC1 activity by direct phosphorylation of Raptor, an activating subunit of mTORC1.

SUMMARY

Alzheimer's disease (AD) is rarely addressed in the context of aging even though there is an overlap in pathology. Phenotypic screens based upon old age-associated brain toxicities were used to develop a potent neurotrophic drug candidate J147. It is shown herein that J147 is effective

against both brain aging and AD-associated pathology in rapidly aging SAMP8 mice, a model for sporadic AD and dementia. An inclusive and integrative multiomics approach was used to investigate protein and gene expression, metabolite levels, and cognition in old and young SAMP8 mice. J147 not only reduced cognitive deficits and associated metabolic changes observed in old SAMP8 mice, it restored multiple markers associated with human AD, vascular pathology, impaired synaptic function, and inflammation to those approaching the young phenotype. Therefore J147 and related compounds (such as CAD-031) can be used to treat or prevent old age-related brain toxicity, AD-associated pathology, and many metabolic aspects of aging.

It is also shown herein that the high affinity molecular target for J147 is mitochondrial ATP synthase (ATPsyn) that also forms the major subunits of the mitochondrial transition pore (MTP). The signaling cascade initiated by the binding of J147 to this target was determined using both the HT22 mouse hippocampal cell line and a human cortical nerve cell line that conditionally expresses the amyloid beta (A β) protein. These data demonstrate that the neuroprotective effect of J147 is mediated through Ca²⁺ signaling. Because of the broadly neuroprotective properties of J147 in both *in vitro* and *in vivo* models of neurodegenerative disease, in part by inhibiting the MPTP, thereby preventing cell death (apoptosis), modulating ATPsyn/MTP can be used to treat these maladies.

Based on these observations, provided are methods of treating or preventing one or more signs of aging in a subject. Such methods can include administering a therapeutic amount of one or more compounds disclosed herein (such as J147 or CAD031) to the subject. Additional agents that can treat or prevent one or more signs of aging can also be administered at therapeutic amounts. In some examples, the subject administered one or more compounds disclosed herein (such as J147 or CAD031) to treat or prevent one or more signs of aging does not have Alzheimer's disease. In some examples, the one or more signs of aging is kidney disease or kidney failure, such as chronic kidney disease and diabetic neuropathy.

In some examples, the methods decrease cytoskeleton-associated protein (Arc), decreases synapse-associated protein 102 (SAP102), decrease phosphorylation of eukaryotic initiation factor 2a (eIF2 α), increase the amount of eIF2 α , decrease the amount of heat shock protein 60 (HSP60), increase the amount of HSP90, decrease the amount of amyloid precursor protein (APP), decrease the amount of APP fragment C99, decrease the amount of APP fragment C83, decrease the level of A β _{1-40S}, decreases the level of total tau protein, decrease levels of tau protein phosphorylation at Ser396, decrease the level of vascular cell adhesion molecule 1 (VCAM-1), decreases the level of endogenous immunoglobulin G (IgG), decrease glial fibrillary acidic protein (GFAP) expression, decrease activation of the stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK), decrease upregulation (e.g., reduces expression) of inflammatory genes such as mitogen-activated

protein kinase (MAPK) kinases, such as Map3k7 and Map2k4), increase expression of *Flt1* that encodes the vascular endothelial growth factor receptor 1, increase levels of docosahexaenoic acid (DHA), increase levels of adrenic acid, increase anti-oxidant effects (*e.g.*, by increasing the AA-metabolites 9-HETE and 8-iso-15-keto PGF₂ β ; the DHA-metabolites 11- and 13-HDoHEs; and the LA-metabolites 9-HODE and 13-HODE), reverse levels of cytochrome P450 metabolites 19-HETE and 20-HETE (AA derivatives), 19(20)-EpDPE (DHA derivative) and 9,10-DiHOME (LA derivative) that occur with aging, reduce levels of TXB₂, reverse levels of 20-HETE and 9,10-DiHOME that occur with aging, increase levels of γ -glutamyl amino acids, increase levels of branched-chain amino acids (BCAAs), increase levels of dipeptides, decrease levels of acylcarnitines, decrease levels of PUFAs, decrease levels of cAMP, reverse levels of glycolytic and TCA intermediates indicative of mitochondrial dysfunction that occur with aging, increase the level of glutamate, increase the level of α -ketoglutarate, decrease levels or expression of mTor, reverse levels of molecules listed in Tables 1 and 2 that occur with aging, or combinations thereof. In some examples, such increases or decreases are at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 75%, at least 80%, at least 90%, or at least 95% as compared to no administration of the one or more compounds.

In some examples, the methods increase kidney weight, decrease TNF-alpha expression, decrease 12-Lox expression, decrease cleaved caspase 1 protein, decrease p65 expression, decrease iNOS expression, or combinations thereof. In some examples, such increases or decreases are at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 75%, at least 80%, at least 90%, or at least 95% as compared to no administration of the one or more compounds.

For example, such methods can be used to promote longevity (*e.g.*, of an older subject), for example by slowing down one or more signs of aging, such as one or more of: compromised BBB homeostasis, decreased brain vascular function, increased brain inflammation, and a pro-oxidant status of the brain.

Also provided herein are methods of identifying an agent that can be used to treat or prevent a neurodegenerative disease, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), glaucoma, retinal degeneration, macular degeneration, age-related hearing loss, mild cognitive impairment, dementia (including, for example, frontotemporal dementia, AIDS dementia, and the like), progressive supranuclear palsy, spinocerebellar ataxias and/or stroke. The methods can include contacting one or more test agents with one or more components of the mitochondrial permeability transition pore (MPTP), such as the mitochondrial ATP synthase (ATPsyn), for example a mitochondrial ATP synthase alpha subunit. In one example, the one or more components of the MPTP includes a J147 binding site.

Such contact can be *in vitro*, for example by contacting the one or more test agents to a cell (such as a neural cell) expressing the one or more components of the MPTP, or *in vivo*, for example by administering the one or more test agents to a non-human mammal, such as a laboratory rodent or non-human primate. Subsequently, the method includes assaying for one or more of MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, mitochondrial calcium levels, or combinations thereof. Agents that alter the MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels by at least 10% as compared to the MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels in an absence of the one or more test agents can be selected for further study. For example agents that increase AMPK activity, ATP production, NAD⁺ production, NADH production, and/or NADPH production by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 200%, at least 300%, at least 400% or at least 500%, as compared to the AMPK activity, ATP production, NAD⁺ production, NADH production, and/or NADPH production, in an absence of the test agent, can be selected. In addition, agents that decrease MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, and/or mitochondrial calcium levels by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, as compared to the MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, and/or mitochondrial calcium levels, in an absence of the test agent, can be selected.

Also provided are methods of treating or preventing a neurodegenerative disease in a subject. Such methods can include administering a therapeutic amount of one or more agents that modulate (*e.g.*, increase or decrease) ATPsyn activity, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels, for example in a neural cell of the subject. For example, agents that increase AMPK activity, ATP production, NAD⁺ production, NADH production, and/or NADPH production by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 200%, at least 300%, at least 400% or at least 500%, as compared to the AMPK activity, ATP production, NAD⁺ production, NADH production, and/or NADPH production in an absence of the agent, can be used. In addition, agents that decrease MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, and/or mitochondrial calcium levels by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%,

at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, as compared to the MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, and/or mitochondrial calcium levels in an absence of the agent, can be used. In one example, the agent is one identified using the methods provided herein. Such methods can further include
 5 administering a therapeutic amount of an additional agent that can treat or prevent a neurodegenerative disease (such as J147).

The foregoing and other objects and features of the disclosure will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: Body weights of SAMP8 mice fed with vehicle and J147 diets. Three-month old SAMP8 mice were fed with vehicle (n = 17) or J147 (n = 18) diets until ten months old. Six SAMP8 mice fed with vehicle diet and two SAMP8 mice fed with J147 diet died during the course
 15 of this study. No significant differences were found between the body weights of the two groups. Two-way repeated measures ANOVA and post hoc Bonferroni corrected t-test. All data are mean $\pm$ SD.

FIGS. 2A-2G: J147 improves locomotor and cognitive function in old SAMP8 mice. Distance travelled (A), average velocity (B), number of jumps (C) and number of vertical events
 20 (D) were assessed in young mice and old SAMP8 mice fed with control or J147 diets with the open field test. The elevated plus maze (E) was used to measure anxiety levels. Recognition memory and spatial learning/memory were evaluated by the object recognition (F) and the Barnes maze (G) assays, respectively. One-way ANOVA followed by Tukey-Kramer post-hoc test and two-way repeated measures ANOVA and post hoc Bonferroni corrected t-test (n = 12-16/group). All data are
 25 mean $\pm$ SD.

FIGS. 3A-3H: Dysregulation of neuronal homeostasis and stress responses in the hippocampus of old SAMP8 mice is partially restored by J147. RIPA-soluble fractions from hippocampal tissue were analyzed by Western blotting for relevant markers of neuronal homeostasis and stress and are presented relative to actin or the unphosphorylated molecule: Arc
 30 (A), SAP102 (B), eIF2 α (C), pEIF2 α (D), HSP40 (E), HSP60 (F), HSP70 (G), HSP90 (H). One-way ANOVA followed by Tukey-Kramer post-hoc test (n = 6/group). All data are mean $\pm$ SD.

FIGS. 4A-4E: J147 prevents alterations in A β and tau homeostasis in the hippocampus of old SAMP8 mice. (A and B) Western blot analysis of APP processing in hippocampal tissue using an antibody against the C-terminus of APP. Full-length APP and the APP cleavage products

C99 and C83 were detected. (C) ELISA for A β . Western blot analysis of total Tau (D) and pTau Ser396 (E). One-way ANOVA followed by Tukey-Kramer post-hoc test (n = 6/group). All data are mean $\pm$ SD.

FIGS. 5A-5G: Increased inflammation and gliosis in the hippocampus of old SAMP8

mice are prevented by J147. Western blot analysis of the marker for vascular endothelial inflammation VCAM-1 (A) and of the IgG (Heavy + Light chains) content (B). (C) Astrocytosis, measured by Western blot of GFAP levels. One-way ANOVA followed by Tukey-Kramer post-hoc test (n = 6/group). (D) Microgliosis was assessed by immunohistochemical (IHC) staining and number of Iba-1-positive cells per mm² of total hippocampus calculated. Original magnification: x100. One-way ANOVA followed by Tukey-Kramer post-hoc test (n = 8/group). (E and F) Activation of the stress/inflammation-associated SAPK/JNK was measured by Western blot analysis of its phosphorylation at Thr183/Tyr185. One-way ANOVA followed by Tukey-Kramer post-hoc test (n = 6/group). All data are mean $\pm$ SD. (G) Quantitative RNA analysis of altered genes related to inflammation. Heatmap and hierarchical clustering of scaled gene expression with respective fold changes and *P* values for the comparisons Old/Young and Old+J147/Old. Scaled expression value (Z-score) is plotted in red–blue color scale with red indicating high expression and blue indicating low expression. One-way ANOVA followed by Tukey-Kramer post-hoc test (n = 3-4/group).

FIG. 6: Changes in eicosanoid metabolism of fatty acids in the cortex of young

SAMP8, old SAMP8 and old SAMP8 mice fed with J147. Significant changes in the metabolites of arachidonic acid, docosaheptaenoic acid, linoleic acid and adrenic acid derived from the actions of COX and cytochrome P450 and non-enzymatic oxidation. One-way ANOVA followed by Tukey-Kramer post-hoc test (n = 5/group). Values are expressed as box-and-whisker plots.

FIGS. 7A-7G: Metabolomic analysis of plasma and cortex demonstrate that

alterations in biological pathways between young SAMP8 and old SAMP8 mice are partially rescued by J147. Plasma (A) and cortex (B) heatmaps of the biochemicals found significantly modified, organized by major biological groups. Scaled expression value (Z-score) is plotted in red–blue color scale with red indicating high expression and blue indicating low expression. Venn diagrams illustrating shared and uniquely affected metabolites in plasma (C) and cortex (D). Correlation of metabolite levels altered in Young/Old and Old+J147/Old in plasma (E) and cortex (F) (units are -log(fold change)). (G) Selection of relevant biochemicals changed between young and old SAMP8 mice affected by J147, including γ -glutamyl amino acids, dipeptides, BCAAs, acyl carnitines and PUFAs in the plasma, and metabolites related to neurotransmission and energetic

pathways in the cortex. One-way ANOVA followed by Tukey-Kramer post-hoc test ($n = 5/\text{group}$). Values are expressed as box-and-whisker plots.

FIGS. 8A-8F: Functional analysis of metabolites found significantly altered.

Biochemicals altered in the plasma (A) and cortex (D) between young and old SAMP8 mice and between old and old SAMP8 mice treated with J147 were organized by descending order according to the number of changes per biological group. In order to provide insight onto the possible biological pathways and diseases/functions associated with the metabolic alterations, Ingenuity Pathway Analysis (IPA) was carried out with the plasma (B and C) and cortex (E and F) metabolites present in the HMDB. Only the top significant pathways are indicated.

FIGS. 9A-9E: Whole transcriptome analysis of hippocampus shows a rescue of some age-related changes in RNA expression by J147. (A) Venn diagram illustrating shared and uniquely affected genes. (B) Heatmap of the 150 genes found significantly modified between Old, Old+J147 and Young SAMP8 mice. Scaled expression value (Z-score) is plotted in yellow–blue color scale with yellow indicating high expression and blue indicating low expression. ($n = 3-4/\text{group}$). (C) Correlation of gene expression altered in Young/Old and Old+J147/Old (units are $-\log(\text{fold change})$). Predicted canonical biological pathways (D) and diseases/functions (E) associated with the alterations in gene expression. Only the top significant pathways are indicated.

FIG. 10 shows measurement of AMPK and mTOR phosphorylation by Western blotting, in young, old and old mice treated with J147.

FIGS. 11A-11C: Target identification by DARTS and affinity precipitation pull-downs. (A) DARTS reveals HSP60 and ATP5A as putative direct J147 targets. The band preserved among J147-treated samples (arrow) indicating direct target engagement. (B) Affinity precipitations with a biotinylated derivative of J147 (BJ147) pulls down an enriched fraction of mitochondrial-associated proteins. (C) Affinity precipitation using subventricular zone (SVZ) lysates from adult mice demonstrates BJ147 binding to ATP5A. Unlabeled J147 competed off ATP5A binding to BJ147.

FIGS. 12A-12B: J147 dampens ionophore-mediated Ca^{2+} influx. J147 reduces Ca^{2+} -influx into both the cytosol (A) and mitochondria (B) when treated with ionophores ionomycin and A23187. $*p < .05$, $**p < .01$ (paired t-test).

FIGS. 13A-13I: J147 targets mitochondrial metabolism. (A) Immunofluorescent confocal microscopy using a coumarin derivative of J147 (CJ147) shows a mitochondrial staining pattern. Nuclei/Nunc647, CJ147. (B) BJ147 localizes to mitochondria in HT22 cells within 10' of addition to tissue culture media. DAPI, Nuclei, BJ147, COXIV. Scale bar = 10 μm . (C) J147 inhibition of ATP synthase from isolated bovine heart mitochondria. $*p < .05$, $**p < .01$ (one-way

ANOVA). (D) Dose-dependent increase of mitochondrial membrane potential ($\Delta\psi_m$) in HT22 cells following J147 treatment. Statistics reported for J147 treatments compared to vehicle. * $p < .05$, ** $p < .001$ (one-way ANOVA, multiple comparisons). (E) ATP5A knockdown phenocopies J147 effect on $\Delta\psi_m$. ** $p < .01$ (unpaired t-test). (f) J147 dose-dependent increase in mitochondrial superoxide production in HT22 cells. siRNA knockdown of ATP5A revealed a similar increase in mitochondrial superoxide production. ** $p < .01$ (unpaired t-test) for vehicle control siRNA compared to vehicle ATP5A siRNA. Remaining statistical comparisons performed between vehicle and J147 treatment within control siRNA or ATP5A siRNA conditions. * $p < .05$, ** $p < .01$, **** $p < .0001$ (one-way ANOVA, multiple comparisons). (G) J147 induces NF- κ B activation in HT22 cells as measured by luciferase reporter activity. Each point represents a separate experiments, each performed in triplicate. * $p < .05$, ** $p < .001$ (unpaired t-test). (H) J147 increases whole cell ATP levels in HT22 cells. Oligomycin and FCCP served as positive and negative controls, respectively. * $p < .05$ (one-way ANOVA, multiple comparisons). (I) J147 increases NAD⁺ levels in unstressed conditions, and partially preserves NAD⁺ and NADPH levels in a model of IAA-induced ischemia in HT22 cells. * $p < .05$, ** $p < .01$ (unpaired t-test).

FIGS. 14A-14H: Knockdown of ATP5A phenocopies the neuroprotective effects of J147. (A) ATP5A knockdown efficiency in MC65 cells (left, western blot). J147 and ATP5A knockdown protect MC65 cells from death in a proteotoxicity model of A β (right). (B) ATP5A knockdown efficiency in HT22 cells. (C) ATP5A knockdown protects HT22 cells from cell death in a model of IAA-induced ischemia. **** $p < .001$ (unpaired t-test). (D) ATP5A knockdown protects HT22 cells from cell death in models of glutamate-induced oxytosis. **** $p < .0001$ (unpaired t-test) (E) ATP5A knockdown does not provide an additive effect to J147-induced protection during oxytosis toxicity. F. J147 maintains levels of ATP during protection from IAA-induced ischemia. (G) J147 mitigates toxic Ca²⁺ accumulation in the mitochondria (Rhod-2) and cytosol (Fluo-4) during glutamate (E)-induced oxytosis. * $p < .01$, ** $p < .001$ (one-way ANOVA, multiple comparisons). (H) ATP5A knockdown reduces toxic mitochondrial Ca²⁺ influx in HT22 cells in during glutamate-induced oxytosis. $p < .01$, $p < .001$ (one-way ANOVA, multiple comparisons).

FIGS. 15A-15D: J147 modulates AMPK/mTOR signaling. Time-course of J147 activation of the AMPK/mTOR signaling pathway. Increasing phosphorylation of AMPK (α -Thr172), Raptor (Ser792), ACC1 (Ser79) and decreasing phosphorylation of S6 (Ser235/236) in HT22 cells MC65 cells (A, B), and primary rat cortical neurons (C, D). Corresponding quantification graphs for AMPK/mTOR targets assayed are below their respective western blot. C,

D. The CamKK2 inhibitor STO-609 attenuated J147-induced activation of AMPK (dotted line vs. 2nd solid line from top) in cortical neurons.

FIGS. 16A-16F: ATP5A knockdown mimics J147-activation of AMPK/mTOR signaling. (A) ATP5A knockdown phenocopies J147 effect on AMPK/mTOR pathway in HT22 cells. Increases in phosphorylation of AMPK (α -Thr172), Raptor (Ser792), ACC1 (Ser79) and decreases phosphorylation of S6 (Ser235/236) in MC65 cells. (B-F) Corresponding quantifications for each target are shown. graphs for AMPK/mTOR targets assayed in (A). * $p < .05$, ** $p < .01$, *** $p < .001$ (one-way ANOVA).

FIGS. 17A-17B: J147 modulates resting Ca²⁺ homeostasis. J147 increases levels of cytosolic Ca²⁺ (A) and decreases mitochondria Ca²⁺ (B) in HT22 cells. Ionomycin and A23187 were used as positive controls. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$ (one-way ANOVA, multiple comparisons).

FIGS. 18A-18F: J147 attenuates age-associated decline and extends lifespan in vivo. (A, B) Old (10 months) SAMP8 mice treated with J147 exhibit increased phosphorylation of AMPK and ACC1 in hippocampal lysates similar to young (3 months) SAMP8 mice. Corresponding bar graphs are shown. * $p < .05$, ** $p < .01$ (one-way ANOVA). C-E. J147 suppresses age-associated transcriptional (C) and metabolomic (D) drift. Specific drift suppression of processes involving carnitine metabolism and ATP synthesis-coupled proton transport were significant (E). Significant suppression of metabolomic drift was observed in plasma metabolites (D). (F) 0.1 and 2 μ M of J147 increases longevity in male drosophila by 9.5% (red line) and 12.8% (purple line), respectively. ** $p < .01$, *** $p < .001$.

FIG. 19: J147-mediated activation of CamKK2 results in the regulation of AMPK/mTOR signaling J147 and siRNA-mediated knockdown of ATP5A modulate activity of the ATP synthase complex, resulting in an increase in cytosolic Ca²⁺ and activation of CamKK2. When activated, CamKK2 phosphorylates and activates AMPK, leading to inhibition of mTOR. Modulating the AMPK/mTOR pathway via ATP synthase has been shown to affect aging and aging-associated neurotoxicities, thereby promoting lifespan extension and protection from toxic stresses.

FIGS. 20A-20C show transcriptomic drift analysis from (A) whole transcriptome analysis of brain (B) metabolomic analysis of plasma and (C) metabolomic analysis of brain.

FIG. 21 is a graph showing that the J147 derivative CAD031 extends the lifespan of SAMP8 mice when given at advanced age (8 months old) (n=23-25).

FIGS. 22A-22D are bar graphs showing the effect of J147 treatment on time-dependent changes in the kidney of aging mice, (A) weight, (B) TNF-alpha expression, (C) 12-Lox

expression, and (D) caspase 1 expression. Data are mean $\pm$ SEM of N=10 /group. Statistical analysis by one way ANOVA with Tukey's post hoc test.

FIGS. 23A-23F are bar graphs showing the effect of CMS-121 treatment on time-dependent changes in the kidney of aging mice, (A) kidney weight, (B) TNF-alpha expression, (C) 12-Lox expression, (D) P65 expression, (E) cleaved caspase 1 expression, and (F) iNos expression.

SEQUENCE LISTING

The nucleic and amino acid sequences are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The sequence listing generated on July 25, 2016 (8 kb) and submitted herewith is herein incorporated by reference.

SEQ ID NO: 1 is an exemplary mitochondrial ATPsyn alpha protein sequence.

DETAILED DESCRIPTION

The following explanations of terms and methods are provided to better describe the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure. The singular forms "a," "an," and "the" refer to one or more than one, unless the context clearly dictates otherwise. For example, the term "comprising a cell" includes single or plural cells and is considered equivalent to the phrase "comprising at least one cell." The term "or" refers to a single element of stated alternative elements or a combination of two or more elements, unless the context clearly indicates otherwise. As used herein, "comprises" means "includes." Thus, "comprising A or B," means "including A, B, or A and B," without excluding additional elements. Dates of GenBank® Accession Nos. referred to herein are the sequences available at least as early as July 23, 2015. All references and GenBank® Accession numbers cited herein are incorporated by reference.

Unless explained otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. The materials, methods, and examples are illustrative only and not intended to be limiting.

In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

Administration: To provide or give a subject an agent, such as a therapeutic agent, by any effective route. Exemplary routes of administration include, but are not limited to, oral, injection (such as subcutaneous, intramuscular, intradermal, intraperitoneal, intravenous, and intratumoral),
5 sublingual, rectal, transdermal, intranasal, vaginal and inhalation routes.

Agent: Any substance or any combination of substances that is useful for achieving an end or result; for example, a substance or combination of substances useful for treating or preventing aging or a neurodegenerative disease. Agents include, and are not limited to, proteins, nucleic acid molecules, compounds, small molecules, organic compounds, inorganic compounds, or other
10 molecules of interest. In some embodiments, the agent is a polypeptide agent (such as an antibody), or a pharmaceutical compound. The skilled artisan will understand that particular agents may be useful to achieve more than one result.

5' AMP-activated protein kinase (AMPK): EC 2.7.11.31. An enzyme that plays a role in cellular energy homeostasis. It includes three proteins (subunits; α , β , and γ) that together make a
15 functional enzyme. Effects of AMPK activation can include stimulation of hepatic fatty acid oxidation and ketogenesis, inhibition of cholesterol synthesis, lipogenesis, and triglyceride synthesis, inhibition of adipocyte lipolysis and lipogenesis, stimulation of skeletal muscle fatty acid oxidation and muscle glucose uptake, and modulation of insulin secretion by pancreatic beta-cells. In some examples, activation of AMPK can be used to treat or prevent a neurodegenerative disease.

Animal: Living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals. Similarly,
20 the term "subject" includes both human and veterinary subjects.

Contacting: Placement in direct physical association; includes both in solid and liquid form, which can take place either *in vivo* or *in vitro*. Contacting includes contact between one
25 molecule and another molecule, for example between a protein and a test agent. Contacting can also include contacting a cell or tissue, for example by placing a test agent in direct physical association with a cell or tissue (such as a hippocampus sample).

Control: A reference standard. In some embodiments, the control is a result expected in the absence of a test agent (such as no substantial effect on MPTP (*e.g.*, amount or activity),
30 ATPsyn activity, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels, for example in a neural cell). In some embodiments, the control is a result expected in the present of an agent that modulates, such as increases, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, and/or NADPH production, or decreases MPTP (*e.g.*, amount or activity), ATPase

activity, ATPsyn activity, and/or mitochondrial calcium levels, for example in a neural cell (such as a hippocampal cell). In still other embodiments, the control is a historical control or standard reference value or range of values (such as a previously tested control sample, such as a group of patients with known prognosis or outcome, or group of samples that represent baseline or normal values).

A difference between a test sample and a control can be an increase or conversely a decrease. The difference can be a qualitative difference or a quantitative difference, for example a statistically significant difference. Suitable statistical analyses are well known in the art, and include, but are not limited to, Student's T test and ANOVA assays. In some examples, a difference is an increase or decrease, relative to a control, of at least about 5%, such as at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 100%, at least about 150%, at least about 200%, at least about 250%, at least about 300%, at least about 350%, at least about 400%, at least about 500%, or greater than 500%.

Detecting, Determining or Measuring: To identify the existence, presence, or fact of something. General methods of detecting are known to the skilled artisan (see, for example, U.S. Patent No. 7,635,476) and may be supplemented with the protocols and reagents disclosed herein. For example, included herein are methods of detecting AMPK activity, ATP production, NAD⁺ production, and/or NADP⁺ production, for example in a neural cell or in a subject. These terms refer to measuring a quantity or quantitating a target molecule in the sample, either absolutely or relatively. Generally, detecting, measuring or determining a biological molecule requires performing an assay, such as mass spectrometry, immunoprecipitation, Western blotting and the like, and not simple observation.

Isolated: An "isolated" biological component (such as a cell, for example a B-cell, a nucleic acid, peptide, protein, heavy chain domain or antibody) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, such as, other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids and polypeptides which have been "isolated" thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and polypeptides prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

Mammal: This term includes both human and non-human mammals. Similarly, the term “subject” includes both human and veterinary subjects (such as cats, dogs, cows, and pigs) and rodents (such as mice and rats).

Mean and Standard Deviation: The *arithmetic mean* is the "standard" average, often simply called the "mean".

$$\bar{x} = \frac{1}{n} \cdot \sum_{i=1}^n x_i$$

The mean is the arithmetic average of a set of values.

The **standard deviation** (represented by the symbol sigma, σ) shows how much variation or "dispersion" exists from the mean. The standard deviation of a random variable, statistical population, data set, or probability distribution is the square root of its variance. The standard deviation is commonly used to measure confidence in statistical conclusions. Generally, twice the standard deviation is about the radius of a 95% confidence interval. Effects that fall far outside the range of standard deviation are generally considered statistically significant. One of skill in the art can readily calculate the mean and the standard deviation from a population of values.

Mitochondrial ATP synthase (ATPsyn): The human mitochondrial (mt) ATP synthase, or complex V (EC 3.6.3.14) consists of two functional domains: F₁, situated in the mitochondrial matrix, and F_o, located in the inner mitochondrial membrane. Complex V uses the energy created by the proton electrochemical gradient to phosphorylate ADP to ATP. F₁ of ATPsyn comprises five different subunits (three α , three β , and one γ , δ and ϵ) and is situated in the mitochondrial matrix. F_o contains subunits c, a, b, d, F₆, OSCP and the accessory subunits e, f, g and A6L. F₁ subunits γ , δ and ϵ constitute the central stalk of complex V. Subunits b, d, F₆ and OSCP form the peripheral stalk. Protons pass from the intermembrane space to the matrix through F_o, which transfers the energy created by the proton electrochemical gradient to F₁, where ADP is phosphorylated to ATP.

Mitochondrial ATPsyn subunit α : Includes mitochondrial ATP synthase alpha subunit nucleic acid molecules and proteins (*e.g.*, OMIM 164360), and in humans is encoded by the ATP5A1 gene. Three copies of the alpha subunit of the mitochondrial ATP synthase along with three copies of the beta subunit, forms the catalytic core of the F₁ complex. Mitochondrial ATPsyn alpha subunit sequences are publically available, for example from the GenBank® sequence database (*e.g.*, Accession Nos. BAA03531.1, CAA46452.1, and NP_075581.1 (*e.g.*, amino acids 44-553 are the mature peptide of NP_075581.1) provide exemplary protein sequences, while Accession Nos. D14710.1, X65460.1, and NM_023093.1 provide exemplary nucleic acid sequences). An exemplary ATPsyn alpha protein sequence is shown in SEQ ID NO: 1.

One of ordinary skill in the art can identify additional mitochondrial ATPsyn alpha subunit nucleic acid and protein sequences, including mitochondrial ATPsyn alpha subunit variants, such as those having at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 1.

Mitochondrial permeability transition pore (MPT pore or MPTP): A protein pore that is formed in the inner membrane of the mitochondria under certain conditions, such as mitochondrial calcium overload, elevated phosphate concentrations and adenine nucleotide depletion, as well as pathological conditions such as traumatic brain injury and stroke. MPTP opening enables free passage into the mitochondria of molecules of <1.5 kDa including protons. The resulting uncoupling of oxidative phosphorylation leads to ATP depletion, mitochondrial swelling and cell death (*e.g.*, through apoptosis or necrosis).

Mechanistic target of rapamycin (mTOR): (*e.g.*, OMIM 601231) A serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, autophagy, transcription. MTOR belongs to the phosphatidylinositol 3-kinase-related kinase protein family, and fibrosis. **mTOR Complex 1 (mTORC1)** is composed of MTOR, regulatory-associated protein of MTOR (Raptor), mammalian lethal with SEC13 protein 8 (MLST8) and the non-core components PRAS40 and DEPTOR. This complex functions as a nutrient/energy/redox sensor and controls protein synthesis. The activity of mTORC1 is stimulated by insulin, growth factors, serum, phosphatidic acid, amino acids (particularly leucine), and oxidative stress.

mTOR sequences are publically available, for example from the GenBank® sequence database (*e.g.*, Accession Nos. NP_004949.1 and NP_064393.2 provide exemplary protein sequences, while Accession Nos. NM_004958.3 and NM_020009.2 provide exemplary nucleic acid sequences). One of ordinary skill in the art can identify additional mTOR nucleic acid and protein sequences, including mTOR subunit variants, such as those having at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% sequence identity to the sequences provided in these GenBank® numbers.

Neurodegenerative disease: A disease associated with the progressive loss of structure or function of neurons, including death of neurons. Examples of such diseases include one or more of Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), glaucoma, retinal degeneration, macular degeneration, age-related hearing loss, mild cognitive impairment, dementia (including, for example, frontotemporal dementia, AIDS dementia, and the like), progressive supranuclear palsy, spinocerebellar ataxias, stroke and the like.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers of use are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 19th Edition, 1995, describes compositions and formulations suitable for pharmaceutical delivery of the agents herein disclosed.

5 In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids, which include, but are not limited to, water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (e.g., powder, pill, tablet, or capsule forms), conventional non-
10 toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

15 **Preventing or treating a disease:** "Preventing" a disease refers to inhibiting the full development of a disease, for example in a person who is known to have a predisposition to a disease or its effects (such as aging or a neurodegenerative disease). One example of a person with a known predisposition is someone who is at least 50 years old, at least 60 years old, at least 65 years old, or at least 70 years old. Another example of a person with a known predisposition is
20 someone with a history of neurodegenerative disease in the family, or who has been exposed to factors (such as a trauma) that predispose the subject to a condition, such as a neurodegenerative disease. "Treatment" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease, disorder, or condition after it has begun to develop for example by causing the reduction, remission, or regression of a disease, disorder or condition. In some embodiments, treatment refers
25 to reduction in memory loss, increase in memory, increased mobility, reduced pain, increased health, or combinations thereof.

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified peptide preparation is one in which the peptide or protein is more enriched than the peptide or protein is in its natural environment within a cell. In
30 one embodiment, a preparation is purified such that the protein or peptide represents at least 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% of the total peptide or protein content of the preparation.

Sequence identity: The similarity between amino acid sequences is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity

is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are. Homologs or variants of a polypeptide will possess a relatively high degree of sequence identity when aligned using standard methods.

Methods of alignment of polypeptide sequences for comparison are well known in the art.

5 Various programs and alignment algorithms are described in: Smith and Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman and Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85:2444, 1988; Higgins and Sharp, *Gene* 73:237, 1988; Higgins and Sharp, *CABIOS* 5:151, 1989; Corpet et al., *Nucleic Acids Research* 16:10881, 1988; and Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85:2444, 1988. Altschul et al., *Nature Genet.* 6:119,
10 1994, presents a detailed consideration of sequence alignment methods and homology calculations. The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., *J. Mol. Biol.* 215:403, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the internet (along with a description of how to determine sequence identity using this program).

15 Homologs and variants of a protein can be characterized by possession of at least about 75%, for example at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity counted over the full length alignment with the amino acid sequence of interest. Proteins with even greater similarity to the reference sequences will show increasing percentage identities when assessed by this method, such as at least 80%, at least 85%, at least
20 90%, at least 95%, at least 98%, or at least 99% sequence identity. When less than the entire sequence is being compared for sequence identity, homologs and variants will typically possess at least 80% sequence identity over short windows of 10-20 amino acids, and may possess sequence identities of at least 85% or at least 90% or 95% depending on their similarity to the reference sequence. One of skill in the art will appreciate that these sequence identity ranges are provided for
25 guidance only; it is entirely possible that strongly significant homologs could be obtained that fall outside of the ranges provided. Thus, in some examples a mitochondrial ATP synthase alpha subunit protein has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity to any of the protein sequences shown in GenBank® Accession Nos. BAA03531.1, CAA46452.1, and NP_075581.1,
30 wherein the variant has mitochondrial ATP synthase alpha subunit protein activity.

Nucleic acids that “selectively hybridize” or “selectively bind” do so under moderately or highly stringent conditions that excludes non-related nucleotide sequences. In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of

complementarity, nucleotide sequence composition (for example, GC v. AT content), and nucleic acid type (for example, RNA versus DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

5 A specific example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1 x SSC at about 68°C (high stringency conditions). One of skill in the art can readily determine variations on these conditions (e.g., *Molecular Cloning: A Laboratory*
10 *Manual*, 2nd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). Washing can be carried out using only one of these conditions, e.g., high stringency conditions, or each of the conditions can be used, e.g., for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be
15 determined empirically.

Subject: Any mammal, such as humans, non-human primates, pigs, sheep, cows, dogs, cats, rodents and the like which is to be the recipient of the particular treatment, such as treatment with one or more disclosed agents, such as treatment with one or more agents that modulate MPTP (e.g., amount or activity), ATPsyn activity, ATPase activity, AMPK activity, ATP production,
20 NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels. In two non-limiting examples, a subject is a human subject or a murine subject. In some examples, the subject is a human at least 50 years old, at least 55 years old, at least 60 years old, at least 65 years old, at least 70 years old, at least 75 years old, or at least 80 years old. In some examples, the subject has one or more neurodegenerative diseases.

25 **Therapeutic agent:** Used in a generic sense, it includes treating agents, prophylactic agents, and replacement agents.

Therapeutically effective amount or effective amount: A quantity of a specific substance, such as a therapeutic agent, sufficient to prevent, treat (including prophylaxis), reduce and/or ameliorate the symptoms and/or underlying causes of any of a disorder or disease. In some
30 embodiments, a therapeutically effective amount is the amount necessary to reduce or eliminate a symptom of a disease, such as aging or a neurodegenerative disorder. When administered to a subject, a dosage will generally be used that will achieve target tissue concentrations that has been shown to achieve a desired *in vitro* effect.

Under conditions sufficient for: A phrase that is used to describe any environment that permits the desired activity.

Overview

5 It is shown herein that the neuroprotective AD drug candidate J147 functions by interacting with the mitochondrial ATPsynthase/MPTP, resulting in the activation of AMPK and increased levels of ATP, NAD⁺, NADH, and NADPH, J147 was developed on the basis of phenotypic screens of old age associated brain toxicities, including proteotoxicity, energy loss, reduced trophic factor support, oxidative stress and inflammation (Chen et al., PloS one 6, e27865, 2011; Prior et al., ACS Chemical Neuroscience 5:503-513, 2014). J147 reduces *in vivo* markers for all of these conditions in the hippocampus of both fAD mice and in rapidly aging SAMP8 mice (Chen et al., PloS one 6, e27865, 2011; Currais et al., Aging 7:1-19, 2015; Prior et al., Alzheimer's Res. Ther. 5:25, 2013). J147 prevents the expression of genes and metabolites associated with aging (Currais et al., Aging 7:1-19, 2015). It also increases BDNF levels and memory in several rodent assays (Chen et al., PloS one 6, e27865, 2011; Prior et al., Alzheimer's Res. Ther. 5:25, 2013). The animal data can be explained by the signaling pathway initiated by J147. AMPK directly activates CREB, leading to brain derived nerve growth factor (BDNF) production, both of which are observed in mice following J147 treatment (Chen et al., 2011; Prior et al., Alzheimer's Res. Ther. 5:25, 2013). CREB and BDNF are required for memory. J147 also reduces A β and markers for inflammation and oxidative stress (Chen et al., PloS one 6, e27865, 2011; Prior et al., Alzheimer's Res. Ther. 5:25, 2013, Currais et al., Aging 7:1-19, 2015). Finally, J147 enhances mitochondrial function in a model of sporadic AD as evidenced by reducing the levels of acylcarnitines in the blood (Currais et al., Aging 7:1-19, 2015). High levels of acylcarnitines are widely used as markers for impaired mitochondrial function. Together the target and signaling pathways described herein are compatible with the published therapeutic effects of J147 in animals.

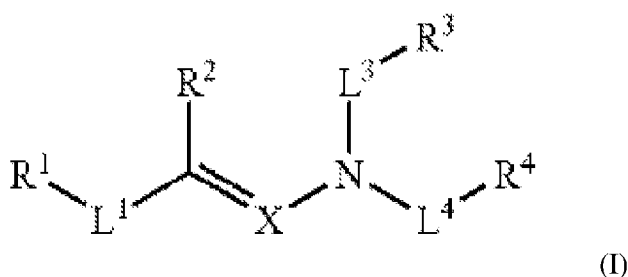
ATPsyn/MPTP, AMPK, IP₃R3, and mTOR are therapeutic drug targets for neurodegenerative disease and aging (Hardie et al., Nat Rev Mol Cell Biol 13:251-262, 2012; Laplante and Sabatini, J. Cell Sci. 122:3589-3594, 2009; Lee et al., Int J Mol Sci 12:5304-5318, 2011); SpteH (Lappano and Maggiolini, Nat Rev Drug Discov 10:47-60, 2011). The data herein show that J147 initially causes an increase in cytoplasmic/mitochondrial Ca²⁺. An increase in matrix Ca²⁺ stimulates dehydrogenases in the citric acid cycle to produce reducing equivalents that ultimately increase ATP production from the respiratory chain (Hajnoczky et al., J Bioenerg Biomembr 32, 15-25, 2000). The effect of IP₃-mediated Ca²⁺ release on mitochondrial membrane potential and cell death pathways is highly cell type dependent. For example, in HEPC2 cells the Ca²⁺ depolarizes

mitochondria and is pro-apoptotic (Szalai et al., EMBO J 18:6349-6361, 1999), while in astrocytes it hyperpolarizes mitochondria and is protective (Wu et al., J. Neurosci. 27:6510-6520, 2007).

High mitochondrial membrane potentials also inhibit the activity of the MTP (Zoratti and Szabo, Biochimica et biophysica acta 1241:139-176, 1995). It is possible that there is a close association
 5 between the ER and the outer mitochondrial membrane that includes IP₃R3 as well as the voltage-dependent anion-selective channel (VDAC) (Hajnóczky and Csordas, Curr Biol 20, R888-891, 2010).

Compounds for Treating Aging

10 The disclosure provides compounds for treating one or more effects of aging, such as J147 or compounds disclosed in US 8,779,002 (herein incorporated by reference). In one example, the compound has the structure shown in Formula I



15 as well as all pharmaceutically acceptable salts, stereoisomers and tautomers thereof, wherein:

R¹ is selected from the group consisting of optionally substituted aryl and optionally substituted heteroaryl;

20 R² is selected from the group consisting of H, optionally substituted alkyl and optionally substituted alkenyl; or

R² is selected from the group consisting of optionally substituted alkylene and optionally substituted alkenylene, such that: R¹ and R², together with L¹ and the carbon to which R² is attached, cooperate to form an optionally substituted bicyclic ring, or when R² and L³ are both optionally substituted alkenylene, R² and L³ cooperate to form an optionally
 25 substituted pyrazole ring;

R³ is selected from the group consisting of optionally substituted alkyl, optionally substituted cycloalkyl, optionally substituted aryl, optionally substituted heteroalkyl, optionally substituted heterocycloalkyl, optionally substituted heteroaryl, optionally

substituted acyl, optionally substituted thioacyl, optionally substituted amino, optionally substituted amido, optionally substituted alkoxy, optionally substituted aryloxy, optionally substituted alkylthio, and optionally substituted arylthio;

R⁴ is selected from the group consisting of optionally substituted cycloalkyl, optionally substituted aryl, optionally substituted heterocycloalkyl and optionally substituted heteroaryl;

X is selected from the group consisting of CR⁵ and N;

R⁵ is selected from the group consisting of H, optionally substituted alkyl and optionally substituted alkenyl; or

R⁵ is selected from the group consisting of optionally substituted alkylene and optionally substituted alkenylene, such that R¹ and R⁵, together with the carbon to which R⁵ is attached, the carbon to which X is attached, and L¹, cooperate to form an optionally substituted bicyclic ring; and

L¹, L³ and L⁴ are independently selected from the group consisting of a covalent bond, optionally substituted alkylene, and optionally substituted alkenylene.

"Alkyl" refers to straight or branched chain alkyl radicals having in the range of about 1 up to about 12 carbon atoms (e.g., methyl, ethyl, propyl, butyl, and the like). "Substituted alkyl" refers to alkyl further bearing one or more substituents (e.g., 1, 2, 3, 4, or even 5) as set forth herein.

"Optionally substituted alkyl" refers to alkyl or substituted alkyl.

"Cycloalkyl" refers to cyclic ring-containing groups containing in the range of about 3 up to about 12 carbon atoms. "Substituted cycloalkyl" refers to cycloalkyl further bearing one or more substituents (e.g., 1, 2, 3, 4, or even 5) selected from alkyl, substituted alkyl, as well as any of the substituents set forth herein. "Optionally substituted cycloalkyl" refers to cycloalkyl or substituted cycloalkyl.

"Heterocycle," "heterocyclic" and like terms refer to cyclic (i.e., ring-containing) groups containing one or more heteroatoms (e.g., N, O, S, or the like) as part of the ring, and having in the range of 1 up to about 14 carbon atoms. "Substituted heterocyclic" and like terms refer to heterocycle further bearing one or more substituents (e.g., 1, 2, 3, 4, or even 5) as set forth herein. Exemplary heterocyclic moieties include saturated rings, unsaturated rings, and aromatic heteroatom-containing ring systems, e.g., epoxy, tetrahydrofuran, oxazoline, pyrrole, pyridine, furan, and the like. "Optionally substituted heterocycle" and like terms refer to heterocycle or substituted heterocycle.

Reference to "optionally substituted bicyclic ring" refers to a bicyclic ring structure as known in the art, optionally including substitutions as defined herein.

"Alkylene" refers to divalent alkyl, and "substituted alkylene" refers to divalent substituted alkyl. Examples of alkylene include without limitation, ethylene ($-\text{CH}_2-\text{CH}_2-$). "Optionally substituted alkylene" refers to alkylene or substituted alkylene.

"Alkene" refers to straight, branched chain, or cyclic hydrocarbyl groups including from 2 to about 20 carbon atoms having at least one, preferably 1-3, more preferably 1-2, most preferably one, carbon to carbon double bond. "Substituted alkene" refers to alkene substituted at 1 or more, e.g., 1, 2, 3, 4, or even 5 positions, with substitution as described herein. "Optionally substituted alkene" refers to alkene or substituted alkene.

"Alkenylene" refers to divalent alkene. Examples of alkenylene include without limitation, ethenylene ($-\text{CH}=\text{CH}-$) and all isomeric forms thereof. "Substituted alkenylene" refers to divalent substituted alkene. "Optionally substituted alkenylene" refers to alkenylene or substituted alkenylene.

"Aryl" refers to aromatic groups having in the range of 6 up to about 14 carbon atoms. "Substituted aryl" refers to aryl radicals further bearing one or more substituents (e.g., 1, 2, 3, 4, or even 5) selected from alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, hydroxyl, alkoxy, aryloxy, mercapto, alkylthio, arylthio, carbonyl, aryl, substituted aryl, heterocyclic, substituted heterocyclic, halogen, trifluoromethyl, pentafluoroethyl, cyano, cyanoalkyl, nitro, amino, amido, amidino, carboxyl, carbamate, SO_2X , wherein X is H, R, NH_2 , NHR or NR_2 , SO_3Y , wherein Y is H, NH_2 , NHR or NR_2 , or $\text{C}(\text{O})\text{Z}$, wherein Z is OH, OR, NH_2 , NHR or NR_2 , and the like.

"Optionally substituted aryl" refers to aryl or substituted aryl.

"Aralkyl" refers to an alkyl group substituted by an aryl group. "Substituted aralkyl" refers to aralkyl further bearing one or more substituents (e.g., 1, 2, 3, 4, or even 5) selected from alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, as well as any of the substituents set forth herein. Thus, aralkyl groups include benzyl, diphenylmethyl, and 1-phenylethyl ($-\text{CH}(\text{C}_6\text{H}_5)(\text{CH}_3)$) among others. "Optionally substituted aralkyl" refers to aralkyl or substituted aralkyl.

"Heteroaryl" refers to aromatic groups containing one or more heteroatoms (e.g., N, O, S, or the like) as part of the aromatic ring, typically having in the range of 2 up to about 14 carbon atoms, and "substituted heteroaryl" refers to heteroaryl radicals further bearing one or more substituents (e.g., 1, 2, 3, 4, or even 5) selected from alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, as well as any of the substituents set forth above.

"Heteroaralkyl" and "heteroarylalkyl" refer to an alkyl group substituted by one or more heteroaryl groups. "Substituted heteroaralkyl" refers to heteroaralkyl further bearing one or more substituents (e.g., 1, 2, 3, 4, or even 5) selected from alkyl, substituted alkyl, cycloalkyl, substituted

cycloalkyl, as well as any of the substituents set forth herein. "Optionally substituted heteroaralkyl" refers to heteroaralkyl or substituted heteroaralkyl.

"Halogen" and "halo" refer to fluorine, chlorine, bromine or iodine.

"Hydroxyl" and "hydroxy" refer to the functionality -OH.

5 "Alkoxy" denotes the group -OR, where R is alkyl. "Substituted alkoxy" denotes the group -OR, where R is substituted alkyl. "Optionally substituted alkoxy" refers to alkoxy or substituted alkoxy.

10 "Aryloxy" denotes the group -OR, where R is aryl. "Substituted aryloxy" denotes the group -OR, where R is substituted aryl. "Optionally substituted aryloxy" refers to aryloxy or substituted aryloxy.

"Mercapto" and "thiol" refer to the functionality -SH.

"Alkylthio" and "thioalkoxy" refer to the group -SR, -S(O)_{n=1-2}-R, where R is alkyl.

15 "Substituted alkylthio" and "substituted thioalkoxy" refers to the group -SR, -S(O)_{n=1-2}-R, where R is substituted alkyl. "Optionally substituted alkylthio" and "optionally substituted thioalkoxy" refers to alkylthio or substituted alkylthio.

"Arylthio" denotes the group -SR, where R is aryl. "Substituted arylthio" denotes the group -SR, where R is substituted aryl. "Optionally substituted arylthio" refers to arylthio or substituted arylthio.

20 "Amino" refers to unsubstituted, monosubstituted and disubstituted amino groups, including the substituent -NH₂, "monoalkylamino," which refers to a substituent having structure -NHR, wherein R is alkyl or substituted alkyl, and "dialkylamino," which refers to a substituent of the structure -NR₂, wherein each R is independently alkyl or substituted alkyl.

"Amidino" denotes the group -C(=NR^q)NR^rR^s, wherein R^q, R^r, and R^s are independently hydrogen or optionally substituted alkyl.

25 Reference to "amide group" embraces substituents of the structure -C(O)-NR₂, wherein each R is independently H, alkyl, substituted alkyl, aryl or substituted aryl as set forth above. When each R is H, the substituent is also referred to as "carbamoyl" (i.e., a substituent having the structure -C(O)-NH₂). When only one of the R groups is H, the substituent is also referred to as "monoalkylcarbamoyl" (i.e., a substituent having the structure -C(O)-NHR, wherein R is alkyl or substituted alkyl as set forth above) or "arylcarbamoyl" (i.e., a substituent having the structure -C(O)-NH(aryl), wherein aryl is as defined above, including substituted aryl). When neither of the R groups are H, the substituent is also referred to as "di-alkylcarbamoyl" (i.e., a substituent having the structure -C(O)-NR₂, wherein each R is independently alkyl or substituted alkyl as set forth above).

30

Reference to "carbamate" embraces substituents of the structure -O-C(O)-NR₇, wherein each R is independently H, alkyl, substituted alkyl, aryl or substituted aryl.

Reference to "ester group" embraces substituents of the structure -O-C(O)-OR, wherein each R is independently alkyl, substituted alkyl, aryl or substituted aryl.

5 "Acyl" refers to groups having the structure -C(O)R, where R is hydrogen, alkyl, aryl, and the like as defined herein. "Substituted acyl" refers to acyl wherein the substituent R is substituted as defined herein. "Optionally substituted acyl" refers to acyl and substituted acyl.

"Cyanoalkyl" refers to the group -R-C≡N, wherein R is optionally substituted alkylene.

Moieties can be substituted with various atoms as described herein. As used here,
 10 "substitution" denotes an atom or group of atoms that has been replaced with another atom or group of atoms (i.e., substituent), and includes all levels of substitution, e.g. mono-, di-, tri-, tetra-, penta-, or even hex-substitution, where such substitution is chemically permissible. Substitutions can occur at any chemically accessible position and on any atom, such as substitution(s) on carbon and any heteroatom, preferably oxygen, nitrogen, or sulfur. For example, substituted moieties include
 15 those where one or more bonds to a hydrogen or carbon atom(s) contained therein are replaced by a bond to non-hydrogen and/or non-carbon atom(s). Substitutions can include, but are not limited to, a halogen atom such as F, Cl, Br, and I; an oxygen atom in groups such as hydroxyl groups, alkoxy groups, aryloxy groups, and ester groups; a sulfur atom in groups such as thiol groups, alkyl and aryl sulfide groups, sulfone groups, sulfonyl groups, and sulfoxide groups; a nitrogen atom in
 20 groups such as amines, amides, alkylamines, dialkylamines, arylamines, alkylarylamines, diarylamines, N-oxides, imides, and enamines; a silicon atom in groups such as trialkylsilyl groups, dialkylarylsilyl groups, alkylarylsilyl groups, and triarylsilyl groups; and heteroatoms in other groups as well known in the art.

Specific exemplary substituents include, without limitation, halogen, -OH, -NH₂, -NO₂, -
 25 CN, -C(O)OH, -C(S)OH, -C(O)NH₂, -C(S)NH₂, -S(O)₂NH₂, -NHC(O)NH₂, -NHC(S)NH₂, -NHS(O)₂NH₂, -C(NH)NH₂, -OR, -SR, -OC(O)R, -OC(S)R, -C(O)R, -C(S)R, -C(O)OR, -C(S)OR, -S(O)R, -S(O)₂R, -C(O)NHR, -C(S)NHR, -C(O)NRR, -C(S)NRR, -S(O)₂NHR, -S(O)₂NRR, -C(NH)NHR, -C(NH)NRR, -NHC(O)R, -NHC(S)R, -NRC(O)R, -NRC(S)R, -NHS(O)₂R, -NRS(O)₂R, -NHC(O)NHR, -NHC(S)NHR, -NRC(O)NH₂, -NRC(S)NH₂, -NRC(O)NHR, -
 30 NRC(S)NHR, -NHC(O)NRR, -NHC(S)NRR, -NRC(O)NRR, -NRC(S)NRR, -NHS(O)₂NHR, -NRS(O)₂NH₂, -NRS(O)₂NHR, -NHS(O)₂NRR, -NRS(O)₂NRR, -NHR, -NRR, where R at each occurrence is independently H, optionally substituted alkyl, optionally substituted aryl, or optionally substituted heteroaryl. Also contemplated is substitution with an optionally substituted hydrocarbyl moiety containing one or more of the following chemical functionalities: -O-, -S-, -

NR-, -O-C(O)-, -O-C(O)-O-, -O-C(O)-NR-, -NR-C(O)-, -NR-C(O)-O-, -NR-C(O)-NR-, -S-C(O)-, -S-C(O)-O-, -S-C(O)-NR-, -S(O)-, -S(O)₂-, -O-S(O)₂-, -O-S(O)₂-O-, -O-S(O)₂-NR-, -O-S(O)-, -O-S(O)-O-, -O-S(O)-NR-, -O-NR-C(O)-, -O-NR-C(O)-O-, -O-NR-C(O)-NR-, -NR-O-C(O)-, -NR-O-C(O)-O-, -NR-O-C(O)-NR-, -O-NR-C(S)-, -O-NR-C(S)-O-, -O-NR-C(S)-NR-, -NR-O-C(S)-, -NR-O-C(S)-O-, -NR-O-C(S)-NR-, -O-C(S)-, -O-C(S)-O-, -O-C(S)-NR-, -NR-C(S)-, -NR-C(S)-O-, -NR-C(S)-NR-, -S-S(O)₂-, -S-S(O)₂-O-, -S-S(O)₂-NR-, -NR-O-S(O)-, -NR-O-S(O)-O-, -NR-O-S(O)-NR-, -NR-O-S(O)₂-, -NR-O-S(O)₂-O-, -NR-O-S(O)₂-NR-, -O-NR-S(O)-, -O-NR-S(O)-O-, -O-NR-S(O)-NR-, -O-NR-S(O)₂-O-, -O-NR-S(O)₂-NR-, -O-NR-S(O)₂-, -O-P(O)R₂-, -S-P(O)R₂-, or -NR-P(O)R₂-, where R at each occurrence is independently H, optionally substituted alkyl, optionally substituted aryl, or optionally substituted heteroaryl.

Disclosed compounds useful in the disclosed methods include isomers including stereoisomers (e.g., enantiomer and diastereomers), constitutional isomers, tautomers, conformational isomers, and geometric isomers.

Exemplary constitutional isomers include for example without limitation, isomers resulting from different connectivity of functionalities forming the compound, for example, 1-propyl versus 2-propyl substitution, and the like. Constitutional isomers in combination with tautomerization additionally embrace bonding rearrangements involving the migration of double bonds and substituents. For example, tautomerization in combination with a 1-3 pleiotropic hydrogen shift, as shown in Scheme 1, results in constitutional isomerism.

Scheme 1.

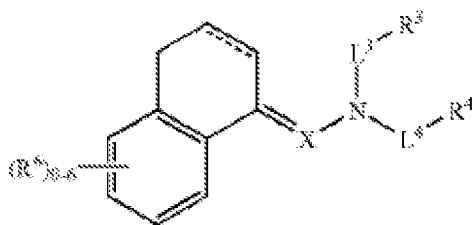


Specifically with respect to the azo compounds produced by tautomerization, also embraced are all pharmaceutically acceptable salts, stereoisomers and any further tautomers thereof, wherein: R¹ is selected from the group consisting of optionally substituted aryl and optionally substituted heteroaryl; R² is selected from the group consisting of H, optionally substituted alkyl and optionally substituted alkenyl; or R² is selected from the group consisting of optionally substituted alkylene and optionally substituted alkenylene, such that R¹ and R², together with L¹ and the carbon to which R² is attached, cooperate to form an optionally substituted bicyclic ring; R⁴ is selected from the group consisting of optionally substituted cycloalkyl, optionally substituted aryl, optionally substituted heterocycloalkyl and optionally substituted heteroaryl; and L¹ and L⁴ are independently

Exemplary conformational isomers include for example without limitation, isomers produced by rotation about a bond wherein the rotation is hindered to the extent that separable isomers result, as well known in the art.

In certain embodiments, R¹ of compounds of Formula (I) is optionally substituted aryl. Exemplary R¹ substituents according to such embodiments include, for example, phenyl, naphthyl, and substituted derivatives thereof. In other embodiments, R¹ is optionally substituted heteroaryl. Exemplary R¹ substituents include, for example, pyridinyl, pyridazinyl, pyrimidyl, pyrazyl, triazinyl, pyrrolyl, pyrazolyl, imidazolyl, (1,2,3)- and (1,2,4)-triazolyl, pyrazinyl, pyrimidinyl, tetrazolyl, furyl, thienyl, isoxazolyl, thiazolyl, phenyl, isoxazolyl, oxazolyl, and substituted derivatives thereof.

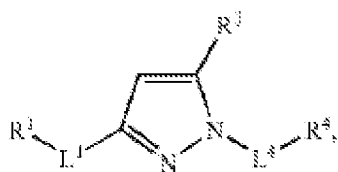
In still other embodiments, R² is selected from the group consisting of optionally substituted alkylene and optionally substituted alkenylene. Accordingly, compounds contemplated by some embodiments have the structure of Formula (Ia):



(Ia)

the symbol represents either a single bond or a double bond.

In certain embodiments, both R^2 and L^3 are optionally substituted alkenylene, and R^2 and L^3 cooperate to form an optionally substituted pyrazole ring, as follows:



5

wherein each of R^1 , R^3 , R^4 , L^1 and L^4 are as previously defined.

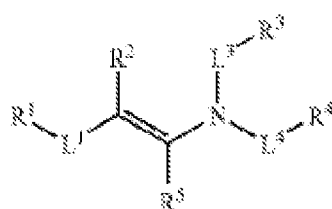
In certain embodiments, compounds having the generic structure set forth above are further defined as follows: R^1 is optionally substituted aryl; R^3 is optionally substituted alkyl; R^4 is optionally substituted aryl; X is N ; and L^1 and L^4 are each a covalent bond.

10

Exemplary compounds according to this embodiment (i.e., compounds wherein R^2 and L^3 cooperate to form an optionally substituted pyrazole ring) include compounds selected from the group consisting of:

Compound No.	R ¹	R ²	R ³	X	L ¹	L ²
A001	3-methoxy phenyl	Trifluoro-methyl	2,4-dimethyl phenyl	N	Covalent bond	Covalent bond
A002	Phenyl	Trifluoro-methyl	Phenyl	N	Covalent bond	Covalent bond
A003	4-methoxy phenyl	Trifluoro-methyl	Phenyl	N	Covalent bond	Covalent bond
A004	Phenyl	Trifluoro-methyl	2,4-dimethyl phenyl	N	Covalent bond	Covalent bond
A005	4-fluoro phenyl	Trifluoro-methyl	Phenyl	N	Covalent bond	Covalent bond
A006	4-methyl phenyl	Trifluoro-methyl	4-sulfonylamino phenyl	N	Covalent bond	Covalent bond
A007	4-sulfonylamino phenyl	Trifluoro-methyl	3,4-dichloro phenyl	N	Covalent bond	Covalent bond
A008	4-nitro phenyl	Trifluoro-methyl	Phenyl	N	Covalent bond	Covalent bond
A009	4-alkoxymethoxy phenyl	Trifluoro-methyl	4-fluoro phenyl	N	Covalent bond	Covalent bond
A010	Phenyl	Trifluoro-methyl	4-amino phenyl	N	Covalent bond	Covalent bond
A011	4-nitro phenyl	Trifluoro-methyl	Phenyl	N	Covalent bond	Covalent bond
A012	4-methyl phenyl	Trifluoro-methyl	phenyl	N	Covalent bond	Covalent bond

In certain embodiments, X=CR⁵, thereby providing compounds having the structure of Formula (Ib):



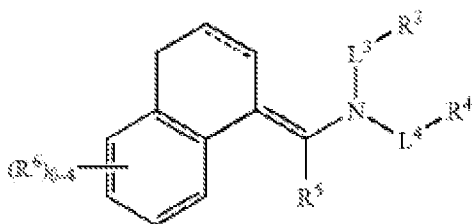
(Ib)

5 wherein R¹, R², R³, R⁴, R⁵, L¹, L² and L³ are as defined for Formula (I). In certain embodiments, R³ is selected from the group consisting of optionally substituted alkyl and optionally substituted acyl. In some embodiments, R⁴ is optionally substituted aryl. In some embodiments, R⁵ is H.

10 In certain embodiments, R⁵ of compounds having the structure of Formula (Ib) is selected from the group consisting of optionally substituted alkyl and optionally substituted alkenyl. In

other embodiments, R^5 is selected from the group consisting of optionally substituted alkylene and optionally substituted alkenylene.

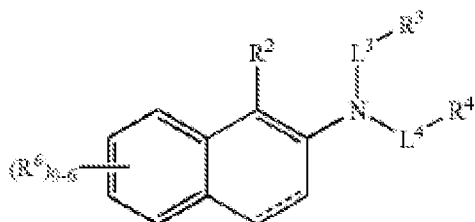
In some embodiments, compounds useful in the disclosed methods have the structure of Formula (Ic):



(Ic)

wherein R^6 at each occurrence is independently selected from the group consisting of alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, hydroxyl, alkoxy, substituted alkoxy, aryloxy, substituted aryloxy, mercapto, alkylthio, arylthio, carbonyl, aryl, substituted aryl, heterocyclic, substituted heterocyclic, halogen, cyano, cyanoalkyl, nitro, amino, amidino, carboxyl, carbamate, $S(O)_nR^7$ and $C(O)R^8$; wherein R^7 is H, R^9 , NH_2 , NHR^9 or NR^9R^{10} , wherein R^8 is OH, OR^9 , NH_2 , NHR^9 or NR^9R^{10} ; R^9 and R^{10} at each occurrence are independently optionally substituted alkyl; $n=1$ or 2; and the symbol $\text{---}\text{---}\text{---}$ represents either a single bond or a double bond.

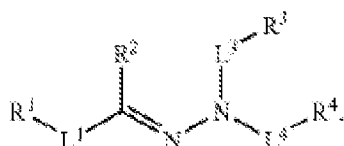
In some embodiments, $X=CR^5$, and R^5 is optionally substituted alkylene or optionally substituted alkenylene, thereby providing compounds having the structure of Formula (Id):



(Id)

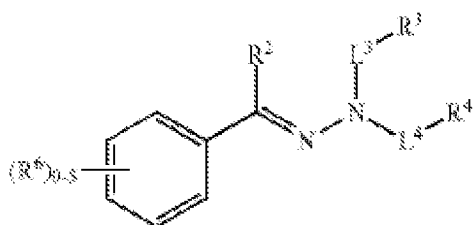
wherein R^6 at each occurrence is independently selected from the group consisting of alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, hydroxyl, alkoxy, substituted alkoxy, aryloxy, substituted aryloxy, mercapto, alkylthio, arylthio, carbonyl, aryl, substituted aryl, heterocyclic, substituted heterocyclic, halogen, cyano, cyanoalkyl, nitro, amino, amidino, carboxyl, carbamate, $S(O)_nR^7$ and $C(O)R^8$; wherein R^7 is H, R^9 , NH_2 , NHR^9 or NR^9R^{10} , wherein R^8 is OH, OR^9 , NH_2 , NHR^9 or NR^9R^{10} ; R^9 and R^{10} at each occurrence are independently optionally substituted alkyl; $n=1$ or 2; and the symbol $\text{---}\text{---}\text{---}$ represents either a single bond or a double bond.

In certain embodiments, X of compounds of Formula (I) is nitrogen, thereby providing compounds having the structure of Formula (Ie):



(Ie)

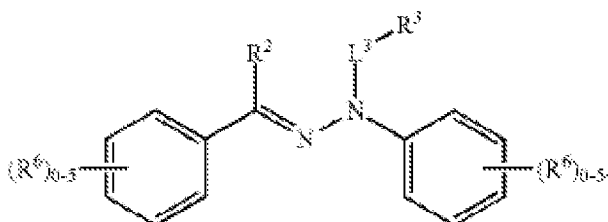
- 5 In certain embodiments, L¹ and R¹ of compounds having the structure of Formula (Ie) can be further defined so as to produce compounds having the structure of Formula (If):



(If)

- wherein R⁶ at each occurrence is independently selected from the group consisting of alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, hydroxyl, alkoxy, substituted alkoxy, aryloxy, substituted aryloxy, mercapto, alkylthio, arylthio, carbonyl, aryl, substituted aryl, heterocyclic, substituted heterocyclic, halogen, cyano, cyanoalkyl, nitro, amino, amidino, carboxyl, carbamate, S(O)_nR⁷ and C(O)R⁸; wherein R⁷ is H, R⁹, NH₂, NHR⁹ or NR⁹R¹⁰, wherein R⁸ is OH, OR⁹, NH₂, NHR⁹ or NR⁹R¹⁰; R⁹ and R¹⁰ at each occurrence are independently optionally substituted alkyl; and n=1 or 2.

- 15 In certain embodiments, compounds having the structure of Formula (Ig) are contemplated:



(Ig)

- or a pharmaceutically acceptable salt, stereoisomer or tautomer thereof. In some examples, R² is selected from the group consisting of H and methyl; R³ is trifluoromethyl or other fluoro substituted alkyl; L³ is a carbonyl; and

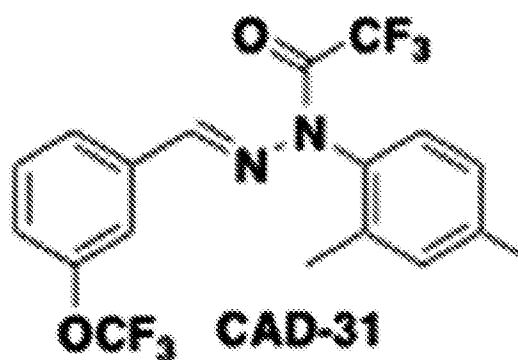
R^6 at each occurrence is independently selected from the group consisting of alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, hydroxyl, alkoxy, substituted alkoxy, aryloxy, substituted aryloxy, mercapto, alkylthio, arylthio, carbonyl, aryl, substituted aryl, halogen, cyano, cyanoalkyl, nitro, amino, amidino, carbamate, $S(O)_nR^7$ and $C(O)R^8$;

5 R^7 is H, R^9 , NH_2 , HNR^9 or NR^9R^{10} ;

R^8 is OH, OR^9 , NH_2 , NHR^9 or NR^9R^{10} ;

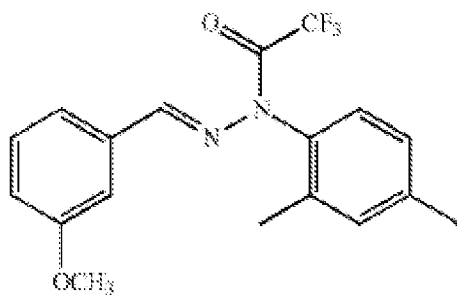
R^9 and R^{10} at each occurrence are independently optionally substituted alkyl; and $n=1$ or 2 .

In one example, the compound for treating one or more effects of aging is



10 (E)-N-(2,4-dimethylphenyl)-2,2,2-trifluoro-N'-(3-(trifluoromethoxy)benzylidene)acetohydrazide (also referred to herein as CAD031).

In certain embodiments, R^2 of compounds having the structure of Formula (Ig) is H. In one example the compound is referred to as compound J147, having the following structure:



15

(J147)

In certain embodiments, L^3 of compounds having the structure of Formula (Ic) is methylene. In certain embodiments, L^4 of compounds having the structure of Formula (Ie) is methylene. In certain embodiments, L^3 of compounds having the structure of Formula (Ie) is ethylene. In certain embodiments, L^4 of compounds having the structure of Formula (Ie) is

20

ethylene. In certain embodiments, L³ of compounds having the structure of Formula (Ie) is ethenylene. In certain embodiments, L⁴ of compounds having the structure of Formula (Ie) is ethenylene.

As recognized by those of skill in the art, compounds useful for the disclosed methods can be readily prepared employing standard synthetic methods. For example, curcumin can be condensed with phenyl hydrazine by warming to reflux overnight in toluene. Optionally, a catalytic amount of acid (HCl) can be employed. Preferably, pure curcumin (vs. technical grade) and freshly distilled phenyl hydrazine will be employed.

As another example, 3-methoxy benzaldehyde can be condensed with 2,4-dimethylphenyl hydrazine in methanol employing standard hydrazone preparation conditions (e.g., heating in the microwave to speed the reaction time). Next, the free NH is acylated with TFAA (trifluoroacetic anhydride) plus catalytic (0.1%) amounts of DMAP (dimethylamino pyridine), THF (tetrahydrofuran) or DCM (dichloromethane).

As yet another example, pyrazoles contemplated by the present disclosure can be prepared by reaction of a suitably substituted 1,3-dione with a suitably substituted hydrazine (e.g., phenylhydrazine). See, for example, J. Med. Chem. 40:3057-63 (1997).

Compounds useful for the disclosed methods can optionally be employed in the form of a composition which includes a compound having the structure of Formula (I) and a pharmaceutically acceptable carrier therefor. In some embodiments, the pharmaceutically acceptable carrier is suitable for oral administration.

In some embodiments, disclosed compounds can optionally be employed in the form of pharmaceutically acceptable salts. "Pharmaceutically acceptable" refers to properties of a compound, including safety, toxicity, and the like, such that a reasonably prudent medical or veterinary practitioner would not be dissuaded from administration of such compound to a subject. Such salts are generally prepared by reacting disclosed compounds with a suitable organic or inorganic acid or base. Representative organic salts include methanesulfonate, acetate, oxalate, adipate, alginate, aspartate, valerate, oleate, laurate, borate, benzoate, lactate, phosphate, toluenesulfonate (tosylate), citrate, malate, maleate, fumarate, succinate, tartrate, napsylate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, benzenesulfonate, butyrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, glucoheptanoate, glycerophosphate, heptanoate, hexanoate, undecanoate, 2-hydroxyethanesulfonate, ethanesulfonate, and the like. Representative inorganic salts can be formed from inorganic acids such as sulfate, bisulfate, hemisulfate, hydrochloride, chlorate, perchlorate, hydrobromide, hydroiodide, and the like. Examples of a base salt include ammonium salts; alkali metal salts such as sodium salts,

potassium salts, and the like; alkaline earth metal salts such as calcium salts, magnesium salts, and the like; salts with organic bases such as dicyclohexylamine salts, N-methyl-D-glucamine, phenylethylamine, and the like; and salts with amino acids such as arginine, lysine, and the like. Such salts can readily be prepared employing methods well known in the art.

5 Provided formulations can include one or more of the above-described compounds and a pharmaceutically acceptable carrier therefor. Exemplary pharmaceutically acceptable carriers include solids, solutions, emulsions, dispersions, micelles, liposomes, and the like. Optionally, the pharmaceutically acceptable carrier employed herein further includes an enteric coating.

10 Pharmaceutically acceptable carriers contemplated for use in the practice of the present disclosure are those which render disclosure compounds amenable to oral delivery, sublingual delivery, transdermal delivery, subcutaneous delivery, intracutaneous delivery, intrathecal delivery, intraocular delivery, rectal delivery, intravenous delivery, intramuscular delivery, topical delivery, nasal delivery, intraperitoneal delivery, vaginal delivery, intracranial delivery, intraventricular delivery, and the like.

15 Thus, formulations can be used in the form of a solid, a solution, an emulsion, a dispersion, a micelle, a liposome, and the like, wherein the resulting formulation contains one or more of the disclosed compounds, as an active ingredient, in admixture with an organic or inorganic carrier or excipient suitable for enterable or parenteral applications. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for
20 tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions and any other suitable for use. The carriers which can be used include glucose, lactose, gum acacia, gelatin, manitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, medium chain length triglycerides, dextrans, and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form. In addition auxiliary, stabilizing, thickening, and
25 coloring agents and perfumes may be used. The active compound(s) is (are) included in the formulation in an amount sufficient to produce the desired effect upon the process or disease condition, such as aging.

 Formulations containing the active ingredient may be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or
30 granules, emulsions, hard or soft capsules, or syrups or elixirs. Formulations intended for oral use may be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such formulations may contain one or more agents selected from the group consisting of a sweetening agent such as sucrose, lactose, or saccharin, flavoring agents such as peppermint, oil of wintergreen or cherry, coloring agents and preserving agents in order to provide

pharmaceutically elegant and palatable preparations. Tablets containing the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients used may be, for example (1) inert diluents such as calcium carbonate, lactose, calcium phosphate or sodium phosphate; (2) granulating and disintegrating agents such corn starch, potato starch or alginic acid; (3) binding agents such as gum tragacanth, corn starch, gelatin or acacia, and (4) lubricating agents such as magnesium stearate, steric acid or talc. The tablets may be uncoated or they may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate may be employed. They may also be coated by such techniques as those described in U.S. Pat. Nos. 4,256,108; 4,160,452; and 4,265,874, to form osmotic therapeutic tablets for controlled release.

In some embodiments, formulations contemplated for oral use may be in the form of hard gelatin capsules wherein the active ingredient is mixed with inert solid diluent(s), for example, calcium carbonate, calcium phosphate or kaolin. They may also be in the form of soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example, peanut oil, liquid paraffin, or olive oil.

Formulations may be in the form of a sterile injectable suspension. This suspension may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides, fatty acids, naturally occurring vegetable oils like sesame oil, coconut oil, peanut oil, cottonseed oil, etc., or synthetic fatty vehicles like ethyl oleate or the like. Buffers, preservatives, antioxidants, and the like can be incorporated as required.

Formulations may also be administered in the form of suppositories for rectal administration of the drug. These formulations may be prepared by mixing the drug with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters of polyethylene glycols, which are solid at ordinary temperatures, but liquefy and/or dissolve in the rectal cavity to release the drug. Since individual subjects may present a wide variation in severity of symptoms and each drug has its unique therapeutic characteristics, the precise mode of administration, dosage employed and treatment protocol for each subject is left to the discretion of the practitioner.

The term "effective amount" as applied to disclosed compounds, means the quantity necessary to effect the desired therapeutic result, for example, a level effective to treat, cure, or

alleviate the symptoms of a disease state for which the therapeutic compound is being administered, or to establish homeostasis. Amounts effective for the particular therapeutic goal sought will, of course, depend upon a variety of factors including the disorder being treated, the severity of the disorder, the activity of the specific compound used, the route of administration, the rate of clearance of the specific compound, the duration of treatment, the drugs used in combination or coincident with the specific compound, the age, body weight, sex, diet and general health of the patient, and like factors well known in the medical arts and sciences. These and other general considerations taken into account in determining the "effective amount" are known to those of skill in the art and are described, e.g., in Gilman et al., eds., Goodman And Gilman's: The Pharmacological Bases of Therapeutics, 8th ed., Pergamon Press, 1990; and Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pa., 1990, each of which is herein incorporated by reference. Effective amounts of disclosed compounds typically fall in the range of about 0.001 up to 100 mg/kg/day; such as in the range of about 0.05 up to 10 mg/kg/day.

Methods of Treating and/or Preventing Aging

The disclosure provides methods for treating or preventing (e.g., reducing the rate of) one or more signs of aging, such as by promoting longevity (e.g., of an older subject), by increasing BBB homeostasis, increasing vascular function (e.g., in the brain), decreasing inflammation (e.g., in the brain), and/or reducing the pro-oxidant status of the brain. In some examples the locomotor function and/or cognitive function of the subject is improved following treatment. Thus, the methods can include administering an effective amount of one or more disclosed compounds to a subject in need thereof, alone or in combination with other therapeutic agents or therapies.

In some examples, the methods decrease cytoskeleton-associated protein (Arc), decreases synapse-associated protein 102 (SAP102), decrease phosphorylation of eukaryotic initiation factor 2a (eIF2 α), increase the amount of eIF2 α , decrease the amount of heat shock protein 60 (HSP60), increase the amount of HSP90, decrease the amount of amyloid precursor protein (APP), decrease the amount of APP fragment C99, decrease the amount of APP fragment C83, decrease the level of A β _{1-40S}, decreases the level of total tau protein, decrease levels of tau protein phosphorylation at Ser396, decrease the level of vascular cell adhesion molecule 1 (VCAM-1), decreases the level of endogenous immunoglobulin G (IgG), decrease glial fibrillary acidic protein (GFAP) expression, decrease activation of the stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK), decrease upregulation (e.g., reduces expression) of inflammatory genes such as mitogen-activated protein kinase (MAPK) kinases, such as Map3k7 and Map2k4), increase expression of *Flt1* that encodes the vascular endothelial growth factor receptor 1, increase levels of docosahexaenoic acid

(DHA), increase levels of adrenic acid, increase anti-oxidant effects (*e.g.*, by increasing the AA-metabolites 9-HETE and 8-iso-15-keto PGF₂ β ; the DHA-metabolites 11- and 13-HDoHEs; and the LA-metabolites 9-HODE and 13-HODE), reverse levels of cytochrome P450 metabolites 19-HETE and 20-HETE (AA derivatives), 19(20)-EpDPE (DHA derivative) and 9,10-DiHOME (LA derivative) that occur with aging, reduce levels of TXB₂, reverse levels of 20-HETE and 9,10-DiHOME that occur with aging, increase levels of γ -glutamyl amino acids, increase levels of branched-chain amino acids (BCAAs), increase levels of dipeptides, decrease levels of acylcarnitines, decrease levels of PUFAs, decrease levels of cAMP, reverse levels of glycolytic and TCA intermediates indicative of mitochondrial dysfunction that occur with aging, increase the level of glutamate, increase the level of α -ketoglutarate, decrease levels or expression of mTor, reverse levels of molecules listed in Tables 1 and 2 that occur with aging, or combinations thereof. In some examples, the level of a particular molecule can be increased or decreased, by altering the activity and/or expression of the molecule. In some examples, such decreases are at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 75%, at least 80%, at least 90%, or at least 95% as compared to no administration of the one or more compounds. In some examples, such increases are at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 75%, at least 80%, at least 90%, at least 100%, at least 200%, at least 300%, at least 400%, or at least 500% as compared to no administration of the one or more compounds. In some examples, such changes by the disclosed compounds occur in the brain, such as the hippocampus.

In some examples, the methods the one or more compounds increase kidney weight, decrease TNF- α expression, decrease 12-Lox expression, decrease cleaved caspase 1 protein, decrease p65 expression, decrease iNOS expression, or combinations thereof. In some examples, such increases in kidney weight are at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 75%, at least 80%, at least 90%, at least 100%, at least 200%, at least 300%, at least 400%, or at least 500% as compared to no administration of the one or more compounds. In some examples, such decreases in expression are at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 75%, at least 80%, at least 90%, or at least 95% as compared to no administration of the one or more compounds.

The subject treated can be any subject, such as a mammal, for example a human or veterinary subject. In some examples, the subject is elderly, such as at least 65 years old, at least 70 years old, at least 75 years old, or at least 80 years old, such as 65- 100 or 65 -90 years old.

Any mode of administration can be used to provide a therapeutically effective amount of a compound to a subject, such as oral, sublingual, intravenous, subcutaneous, transcutaneous, intramuscular, intracutaneous, intrathecal, epidural, intraocular, intracranial, inhalation, rectal,

vaginal, and the like administration. Administration in the form of creams, lotions, tablets, capsules, pellets, dispersible powders, granules, suppositories, syrups, elixirs, lozenges, injectable solutions, sterile aqueous or non-aqueous solutions, suspensions or emulsions, patches, and the like, is also contemplated. The active ingredients can be compounded with non-toxic, pharmaceutically acceptable carriers including, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, dextrans, and the like.

The route of administration will vary with the clinical indication. Some variation in dosage will necessarily occur depending upon the condition of the patient being treated, and the physician will, in any event, determine the appropriate dose for the individual patient. The effective amount of compound per unit dose depends, among other things, on the body weight, physiology, and chosen inoculation regimen. A unit dose of compound refers to the weight of compound employed per administration event without the weight of carrier (when carrier is used). In some examples, the compound is administered at a dose of at least 0.1 mg/kg, at least 1 mg/kg, at least 10 mg/kg, at least 30 mg/kg, at least 100 mg/kg or even least 1 g/kg. In some examples, the compound is administered at a dose of at least 1 mg/day, at least 10 mg/day, at least 50 mg per day, at least 100 mg/day, at least 500 mg/day, at least 1 g/day, or at least 10 g/day.

Targeted-delivery systems, such as polymer matrices, liposomes, microspheres, nanoparticles, and the like, can increase the effective concentration of a therapeutic agent at the site where the therapeutic agent is needed and decrease undesired effects of the therapeutic agent. With more efficient delivery of a therapeutic agent, systemic concentrations of the agent are reduced because lesser amounts of the therapeutic agent can be administered while accruing the same or better therapeutic results. Methodologies applicable to increased delivery efficiency of therapeutic agents typically focus on attaching a targeting moiety to the therapeutic agent or to a carrier which is subsequently loaded with a therapeutic agent.

Various drug delivery systems have been designed by using carriers such as proteins, peptides, polysaccharides, synthetic polymers, colloidal particles (i.e., liposomes, vesicles or micelles), microemulsions, microspheres and nanoparticles. These carriers, which contain entrapped pharmaceutically useful agents, are intended to achieve controlled cell-specific or tissue-specific drug release.

The compounds described herein can be administered in the form of liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of

forming liposomes can be used. The compounds described herein, when in liposome form can contain, in addition to the compounds described herein, stabilizers, preservatives, excipients, and the like. Exemplary lipids are the phospholipids and the phosphatidyl cholines (lecithins), both natural and synthetic. Methods to form liposomes are known in the art. (See, e.g., Prescott, Ed.,
5 Methods in Cell Biology, Volume XIV, Academic Press, New York, N.Y., (1976), p 33 et seq.).

Several delivery approaches can be used to deliver therapeutic agents to the brain by circumventing the blood-brain barrier. Such approaches utilize intrathecal injections, surgical implants (Ommaya, Cancer Drug Delivery, 1: 169-178 (1984) and U.S. Pat. No. 5,222,982), interstitial infusion (Bobo et al., Proc. Natl. Acad. Sci. U.S.A., 91: 2076-2080 (1994)), and the like.
10 These strategies deliver an agent to the CNS by direct administration into the cerebrospinal fluid (CSF) or into the brain parenchyma (ECF).

Drug delivery to the central nervous system through the cerebrospinal fluid can be achieved, for example, by means of a subdurally implantable device the "Ommaya reservoir". The drug is injected into the device and subsequently released into the cerebrospinal fluid surrounding the
15 brain. It can be directed toward specific areas of exposed brain tissue which then adsorb the drug. This adsorption is limited since the drug does not travel freely. A modified device, whereby the reservoir is implanted in the abdominal cavity and the injected drug is transported by cerebrospinal fluid (taken from and returned to the spine) to the ventricular space of the brain, is used for agent administration. Through omega-3 derivatization, site-specific biomolecular complexes can
20 overcome the limited adsorption and movement of therapeutic agents through brain tissue.

Another strategy to improve agent delivery to the CNS is by increasing the agent absorption (adsorption and transport) through the blood-brain barrier and the uptake of therapeutic agent by the cells (Broadwell, Acta Neuropathol., 79: 117-128 (1989); Pardridge et al., J. Pharmacol. Experim. Therapeutics, 255: 893-899 (1990); Banks et al., Progress in Brain Research, 91:139-148
25 (1992); Pardridge, Fuel Homeostasis and the Nervous System, ed.: Vranic et al., Plenum Press, New York, 43-53 (1991)). The passage of agents through the blood-brain barrier to the brain can be enhanced by improving either the permeability of the agent itself or by altering the characteristics of the blood-brain barrier. Thus, the passage of the agent can be facilitated by increasing its lipid solubility through chemical modification, and/or by its coupling to a cationic carrier, or by its
30 covalent coupling to a peptide vector capable of transporting the agent through the blood-brain barrier. Peptide transport vectors are also known as blood-brain barrier permeabilizer compounds (U.S. Pat. No. 5,268,164). Site specific macromolecules with lipophilic characteristics useful for delivery to the brain are described in U.S. Pat. No. 6,005,004.

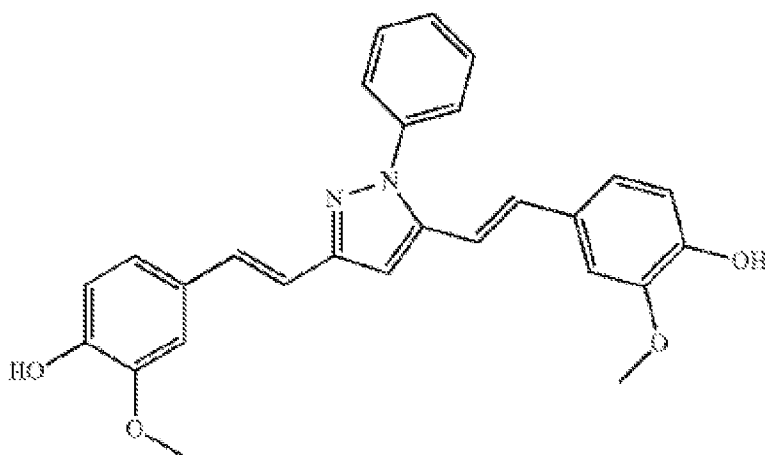
Other examples (U.S. Pat. No. 4,701,521, and U.S. Pat. No. 4,847,240) describe a method of covalently bonding an agent to a cationic macromolecular carrier which enters into the cells at relatively higher rates. These patents teach enhancement in cellular uptake of bio-molecules into the cells when covalently bonded to cationic resins.

5 U.S. Pat. No. 4,046,722 discloses anti-cancer drugs covalently bonded to cationic polymers for the purpose of directing them to cells bearing specific antigens. The polymeric carriers have molecular weights of about 5,000 to 500,000. Such polymeric carriers can be employed to deliver compounds described herein in a targeted manner.

Further work involving covalent bonding of an agent to a cationic polymer through an acid-sensitive intermediate (also known as a spacer) molecule, is described in U.S. Pat. No. 4,631,190
10 and U.S. Pat. No. 5,144,011. Various spacer molecules, such as cis-aconitic acid, are covalently linked to the agent and to the polymeric carrier. They control the release of the agent from the macromolecular carrier when subjected to a mild increase in acidity, such as probably occurs within a lysosome of the cell. The drug can be selectively hydrolyzed from the molecular conjugate
15 and released in the cell in its unmodified and active form. Molecular conjugates are transported to lysosomes, where they are metabolized under the action of lysosomal enzymes at a substantially more acidic pH than other compartments or fluids within a cell or body. The pH of a lysosome is shown to be about 4.8, while during the initial stage of the conjugate digestion, the pH is possibly as low as 3.8.

20 Disclosed compounds, and compounds useful for comparison of aging properties therewith, include the following:

CNB-001



**Method of Identifying Compounds to
Treat or Prevent Neurodegenerative Disease(s)**

Aging is a major driving force for dementia, such as that caused by Alzheimer's disease (AD). A link between aging and dementia is presented through the identification of the molecular target for the AD drug candidate J147. It is shown herein that the mitochondrial α -F1-ATP synthase (ATP5A) is a target for J147. Thus, the mitochondrial ATP synthase is a shared drug target among aging, dementia, and Alzheimer's disease. J147 induces the modulation of ATP synthase activity, alleviates the accumulation of toxic intracellular calcium in neural cells exposed to acute stress, and in mice results in the sustained activation of the AMPK/mTOR pathway, a canonical longevity mechanism. Accordingly, J147 prevents age-associated drift of the transcriptome and metabolome in mice, and extends life span in male drosophila. These findings demonstrate the utility of targeting age-associated brain toxicities in phenotypic screens for AD drug candidates. The results indicate that aging and age-associated dementia share some common molecular drug targets that can potentially be exploited for the suppression of both.

Based on these observations, provided herein are methods of identifying agents that can be used to treat or prevent a neurodegenerative disease, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), glaucoma, retinal degeneration, macular degeneration, age-related hearing loss, mild cognitive impairment, dementia (including, for example, frontotemporal dementia, AIDS dementia, and the like), progressive supranuclear palsy, stroke and/or spinocerebellar ataxias. The methods can include contacting one or more test agents with one or more components of the mitochondrial permeability transition pore (MPTP), such as the mitochondrial ATP synthase (ATPsyn), for example a mitochondrial ATP synthase alpha subunit. In one example, the methods include contacting one or more test agents with a mitochondrial ATP synthase alpha subunit having at least 80%, at least 90%, at least 92%, at least 95%, at least 97%, at least 98%, or at least 99% sequence identity to the amino acid sequence of GenBank Accession No. BAA03531.1, CAA46452.1, or NP_075581.1 or SEQ ID NO: 1. In one example, the methods include contacting one or more test agents with a mitochondrial ATP synthase alpha subunit having or consisting of the amino acid sequence of GenBank Accession No. BAA03531.1, CAA46452.1, or NP_075581.1 or SEQ ID NO: 1. In one example, the one or more components of the MPTP includes a J147 binding site.

Subsequently, the method includes assaying for one or more of MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, mitochondrial calcium levels, or combinations thereof. Thus, the amount of MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, AMPK

activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels can be measured using routine methods. Such measurements can be qualitative or quantitative.

5 In some examples, AMPK activity is measured. In one example, AMPK activity is measured using a commercially available kit, such as the CycLex® AMPK kinase assay kit from MBL International. In one example, AMPK activity is measured by immunoprecipitating AMPK from the cells or tissue of interest in the subject (such as the hippocampus) using appropriate antibodies (such as phospho antibodies specific for AMPK from Cell Signalling Technology), followed by measuring AMPK enzymatic activity using radiolabeled adenosine triphosphate (ATP)
10 in the presence of a suitable substrate.

In some examples, ATP production is measured. In one example, ATP production is measured using a commercially available kit, such as the ATP assay kit from abcam (Cambridge, MA), or the ATP colorimetric/fluorometric kit from BioVision (Milpitas, CA).

15 In some examples, NAD⁺ and/or NADH production is measured. In one example, NAD⁺ and/or NADH production is measured using a commercially available kit, such as the NAD⁺/NADH Assay kit from Abnova (Walnut, CA), or the CycLex® NAD⁺/NADH Colorimetric assay kit from MBL International.

In some examples, NADPH production is measured. In one example, NADPH production is measured using a commercially available kit, such as the NADP/NADPH Assay kit from abcam
20 (Cambridge, MA) or BioVision (Milpitas, CA).

Agents that alter the AMPK activity, ATP production, NAD⁺ production, NADH production, and/or NADPH production, by at least 10% as compared to the AMPK activity, ATP production, NAD⁺ production, NADH production, and/or NADPH production, in an absence of the one or more test agents can be selected for further study. For example agents that increase AMPK
25 activity, ATP production, NAD⁺ production, NADH production, and/or NADPH production, by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 200%, at least 300%, at least 400% or at least 500%, as compared to the AMPK activity, ATP production, NAD⁺ production, NADH production, and/or NADPH production, in an absence of the test agent, can be selected.

30 In some examples, mitochondrial calcium is measured. In one example, mitochondrial calcium is measured using a commercially available kit, such as those from antibodies-online.com (Atlanta, GA).

In some examples, MPTP activity is measured. In one example, MPTP activity is measured using a commercially available kit, such as the MitoProbe™ Transition Pore Assay kit from Molecular Probes (Eugene, OR).

5 In some examples, ATPsyn activity or levels are measured, for example using appropriate antibodies or nucleic acid probes.

Agents that alter MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, and/or mitochondrial calcium levels, by at least 10% as compared to the MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, and/or mitochondrial calcium levels, in an absence of the one or more test agents can be selected for further study. For example agents that decrease MPTP (*e.g.*,
10 amount or activity), ATPsyn activity, ATPase activity, and/or mitochondrial calcium levels, by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or 100%, as compared to MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, and/or mitochondrial calcium levels, in an absence of the test agent, can be selected.

15 Contact between the test agent(s) and the MPTP can be *in vitro*, for example by contacting the one or more test agents to a cell (such as a neural cell, for example a hippocampal cell) expressing the one or more components of the MPTP (or a portion of such a component, such as one that binds J147), or *in vivo*, for example by administering the one or more test agents to a non-human mammal, such as a laboratory rodent or non-human primate. In some examples, *in vitro*
20 assays are performed first, and selected test agent(s) having the desired effect on MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels are administered to a non-human mammal (*e.g.*, rat, mouse, or non-human primate), and the effect on MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, AMPK activity, ATP
25 production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels determined in the mammal. One or more test agents that alter the MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels by at least 10% as compared to the MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity,
30 AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels in an absence of the one or more test agents can be selected.

The amount of MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels can be compared to a control, such as a reference value (or range of

values), in a cell (or subject) not contacted with the agent of interest, or contacted with an agent known not to affect MPTP (*e.g.*, amount or activity), ATPsyn activity, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels. An increase in AMPK activity, ATP production, NAD⁺ production, NADH production, and/or NADPH production indicates that the agent is useful as a therapeutic to treat or prevent a neurodegenerative disease. In some examples, an increase in AMPK activity, ATP production, NAD⁺ production, NADH production, and/or NADPH production is assessed. The increase can be at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 200%, at least 300%, at least 400% or at least 500%, as compared to a control. In some embodiments, a decrease in MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, and/or mitochondrial calcium levels indicates the agent is useful as a therapeutic to treat or prevent a neurodegenerative disease. The decrease can be at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or about 100%, as compared to a control.

In one embodiment, high throughput screening methods are used that involve providing a combinatorial small organic molecule or peptide library containing a large number of potential therapeutic agents (potential modulator or ligand compounds). Such “combinatorial chemical libraries” or “ligand libraries” are then screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional “lead compounds” or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical “building blocks” such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (*i.e.*, the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks, and screened using the disclosed assays.

Preparation and screening of combinatorial chemical libraries to identify therapeutic agents are well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (*see, e.g.*, U.S. Patent 5,010,175, Furka, *Int. J. Pept. Prot. Res.* 37:487-493 (1991) and Houghton *et al.*, *Nature* 354:84-88 (1991)). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (*e.g.*, PCT Publication No. WO 91/19735), encoded peptides (*e.g.*, PCT

Publication No. WO 93/20242), random bio-oligomers (e.g., PCT Publication No. WO 92/00091), benzodiazepines (e.g., U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al.*, *Proc. Nat. Acad. Sci. USA* 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al.*, *J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann *et al.*, *J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen *et al.*, *J. Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho *et al.*, *Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell *et al.*, *J. Org. Chem.* 59:658 (1994)), nucleic acid libraries (*see* Ausubel, Berger and Sambrook, all *supra*), peptide nucleic acid libraries (*see, e.g.*, U.S. Patent 5,539,083), antibody libraries (*see, e.g.*, Vaughn *et al.*, *Nature Biotechnology*, 14(3):309-314 (1996)), carbohydrate libraries (*see, e.g.*, Liang *et al.*, *Science*, 274:1520-1522 (1996) and U.S. Patent 5,593,853), small organic molecule libraries (*see, e.g.*, benzodiazepines, Baum C&EN, Jan 18, page 33 (1993); isoprenoids, U.S. Patent 5,569,588; thiazolidinones and metathiazanones, U.S. Patent 5,549,974; pyrrolidines, U.S. Patents 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent 5,506,337; benzodiazepines, 5,288,514, and the like).

The agents tested can be any small organic molecule, or a biological entity, such as a protein, e.g., an antibody or peptide, aptazyme, aptamer, sugar, nucleic acid, e.g., an antisense oligonucleotide or a ribozyme or siRNA, or a lipid. In some examples, test compounds are small organic molecules, peptides, circular peptides, siRNA, antisense molecules, ribozymes, and lipids.

Essentially any chemical compound can be used as a potential modulator of MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels, although in some examples compounds can be dissolved in aqueous or organic (*e.g.*, DMSO-based) solutions. The assays are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (*e.g.*, in microtiter formats on microtiter plates in robotic assays). It will be appreciated that there are many suppliers of chemical compounds, including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-Aldrich (St. Louis, MO), Fluka Chemika-Biochemica Analytika (Buchs Switzerland) and the like.

Devices for the preparation of combinatorial libraries are commercially available (*see, e.g.*, 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA). In addition, numerous combinatorial libraries are themselves commercially available (*see, e.g.*, ComGenex,

Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, etc.).

Any of the assays disclosed herein can be adapted for high throughput screening. In high throughput assays, either soluble or solid state, it is possible to screen up to several thousand different test agents in a single day. In particular, each well of a microtiter plate can be used to run a separate assay against a test agent, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single test agent. Thus, a single standard microtiter plate can assay about 100 (*e.g.*, 96) modulators. If 1536 well plates are used, then a single plate can easily assay from about 100- about 1500 different test agents. It is possible to assay many plates per day; assay screens for up to about 6,000, 20,000, 50,000, or more than 100,000 different test agents are possible using integrated systems.

Methods of Treating or Preventing a Neurodegenerative Disease

Also provided are methods of treating or preventing a neurodegenerative disease in a subject. Exemplary neurodegenerative diseases that can be treated or prevented with the disclosed methods include, but are not limited to: Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), glaucoma, retinal degeneration, macular degeneration, age-related hearing loss, mild cognitive impairment, dementia (including, for example, frontotemporal dementia, AIDS dementia, and the like), progressive supranuclear palsy, stroke, and/or spinocerebellar ataxias. Thus, subject can have Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), glaucoma, retinal degeneration, macular degeneration, age-related hearing loss, mild cognitive impairment, dementia (including, for example, frontotemporal dementia, AIDS dementia, and the like), progressive supranuclear palsy, suffered a stroke, and/or spinocerebellar ataxias. The subject can be any mammalian subject, including human subjects, laboratory mammals, and veterinary subjects such as cats and dogs. The subject can be a child or an adult. In some examples, the method includes selecting a subject with a neurodegenerative disease, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), glaucoma, retinal degeneration, macular degeneration, age-related hearing loss, mild cognitive impairment, dementia (including, for example, frontotemporal dementia, AIDS dementia, and the like), progressive supranuclear palsy, and/or spinocerebellar ataxias, or a subject at risk for such neurodegenerative disease(s). These subjects can be selected for treatment with one or more agents that modulate (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP

production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels.

Such methods can include administering a therapeutic amount of one or more agents that modulate (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels, for example in a neural cell (such as a hippocampal cell of the subject. For example, agents that increase AMPK activity, ATP production, NAD⁺ production, NADH production, and/or NADPH production by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 200%, at least 300%, at least 400% or at least 500%, as compared to the AMPK activity, ATP production, NAD⁺ production, NADH production, and/or NADPH production in an absence of the agent, can be used. In one example, the agent is one identified using the methods provided herein. For example, agents that decrease MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, and/or mitochondrial calcium levels by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or about 100%, as compared to the MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, and/or mitochondrial calcium levels in an absence of the agent, can be used. In one example, the agent is one identified using the methods provided herein. Such methods can further include administering a therapeutic amount of an additional agent that can treat or prevent a neurodegenerative disease.

The agent(s) that modulates (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels, can be administered to a subject, for example to treat a neurodegenerative disease, for example by reducing the presence or activity of ATPsyn, increasing memory, reducing memory loss (or the rate of such loss), or combinations thereof.

The agent(s) that modulates (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels can be administered to humans or other mammals (such as laboratory mammals, for example mice, rats, chimpanzees, apes, as well as pets, such as dogs and cats) by any means, including orally, intravenously, intramuscularly, intraperitoneally, intranasally, intradermally, intrathecally, subcutaneously, via inhalation or via suppository. In one non-limiting example, the composition is administered via injection. In some examples, site-specific administration of the composition can

be used, for example by administering the agent(s) that modulates (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels to brain tissue (for example by using a pump, or by implantation of a slow release form in the brain). In some examples, the agent(s) that modulates (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels can cross the blood brain barrier. The particular mode of administration and the dosage regimen will be selected by the attending clinician, taking into account the particulars of the case (*e.g.* the subject, the disease, the disease state involved, the particular treatment, and whether the treatment is prophylactic). Treatment can involve daily or multi-daily or less than daily (such as weekly or monthly etc.) doses over a period of a few days to months, or even years. For example, a therapeutically effective amount of the agent(s) that modulates (*e.g.*, increases or decreases) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels can be administered in a single dose, twice daily, weekly, or in several doses, for example daily, or during a course of treatment. In a particular non-limiting example, treatment involves once daily dose or twice daily dose. In some examples, the therapeutic compound is administered at a dose of at least 0.1 mg/kg, at least 1 mg/kg, at least 10 mg/kg, at least 30 mg/kg, at least 100 mg/kg or even least 1 g/kg. In some examples, the compound is administered at a dose of at least 1 mg/day, at least 10 mg/day, at least 50 mg per day, at least 100 mg/day, at least 500 mg/day, at least 1 g/day, or at least 10 g/day.

The amount of agent(s) that modulates (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels administered can be dependent on the subject being treated, the severity of the affliction, and the manner of administration, and can be left to the judgment of the prescribing clinician. Within these bounds, the formulation to be administered will contain a quantity of the therapeutic agent in amounts effective to achieve the desired effect in the subject being treated. These and other general considerations taken into account in determining the "effective amount" are known to those of skill in the art and are described, *e.g.*, in Gilman et al., eds., Goodman And Gilman's: The Pharmacological Bases of Therapeutics, 8th ed., Pergamon Press, 1990; and Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pa., 1990, each of which is herein incorporated by reference. A therapeutically effective amount of an agent(s) that modulates (*e.g.*,

increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels can be the amount of the agent necessary to treat or prevent a neurodegenerative disease (for example a reduction in one or more signs or symptoms of a neurodegenerative disease by at least 5%, at least 10%, at least 20%, at least 50%, or at last 75%, for example relative to no administration of the therapeutic agent). In some examples, a therapeutic amount of agent(s) that modulates (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels is about 0.001 to 100 mg/kg/day; such as about 0.05 to 10 mg/kg/day, 0.05 to 1 mg/kg/day, 0.01 to 1 mg/kg/day, 0.1 to 1 mg/kg/day, 0.1 to 10 mg/kg/day, 1 to 10 mg/kg/day or 1 to 20 mg/kg/day.

Thus, formulations containing one or more agent(s) that modulate (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels can be used in the form of a solid, a solution, an emulsion, a dispersion, a micelle, a liposome, and the like, wherein the resulting formulation contains one or more of the therapeutic agents as an active ingredient, in admixture with an organic or inorganic carrier or excipient suitable for enterable or parenteral applications. The active ingredient may be compounded, for example, with non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions and any other suitable for use. The carriers which can be used include glucose, lactose, gum acacia, gelatin, manitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, medium chain length triglycerides, dextrans, and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form. In addition auxiliary, stabilizing, thickening, and coloring agents and perfumes may be used. The active compound(s) is (are) included in the formulation in an amount sufficient to produce the desired effect upon the neurodegenerative disease.

Formulations containing the one or more agent(s) that modulate (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels may be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsions, hard or soft capsules, or syrups or elixirs. Formulations intended for oral use may be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such formulations may contain one or more agents selected from the group consisting of a sweetening agent such as

sucrose, lactose, or saccharin, flavoring agents such as peppermint, oil of wintergreen or cherry, coloring agents and preserving agents in order to provide pharmaceutically elegant and palatable preparations. Tablets containing the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients used may be, for example (1) inert diluents such as calcium carbonate, lactose, calcium phosphate or sodium phosphate; (2) granulating and disintegrating agents such corn starch, potato starch or alginic acid; (3) binding agents such as gum tragacanth, corn starch, gelatin or acacia, and (4) lubricating agents such as magnesium stearate, steric acid or talc. The tablets may be uncoated or they may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate may be employed. They may also be coated by such techniques as those described in U.S. Pat. Nos. 4,256,108; 4,160,452; and 4,265,874, to form osmotic therapeutic tablets for controlled release.

In some embodiments, formulations contemplated for oral use may be in the form of hard gelatin capsules wherein the active ingredient is mixed with inert solid diluent(s), for example, calcium carbonate, calcium phosphate or kaolin. They may also be in the form of soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example, peanut oil, liquid paraffin, or olive oil.

Formulations containing the one or more agent(s) that modulate (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels can be in the form of a sterile injectable suspension. This suspension may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides, fatty acids, naturally occurring vegetable oils like sesame oil, coconut oil, peanut oil, cottonseed oil, etc., or synthetic fatty vehicles like ethyl oleate or the like. Buffers, preservatives, antioxidants, and the like can be incorporated as required.

Formulations can also be administered in the form of suppositories for rectal administration of the one or more agent(s) that modulate (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels. These

formulations may be prepared by mixing the drug with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters of polyethylene glycols, which are solid at ordinary temperatures, but liquefy and/or dissolve in the rectal cavity to release the drug. Since individual subjects may present a wide variation in severity of symptoms and each drug has its unique therapeutic characteristics, the precise mode of administration, dosage employed and treatment protocol for each subject is left to the discretion of the practitioner.

In some examples, the agent(s) that modulates (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels, is administered in combination (such as sequentially or simultaneously or contemporaneously) with effective amounts of one or more other agents, such as those useful in the treatment or prevention of a neurodegenerative disease. The term “administration in combination” or “co-administration” refers to both concurrent and sequential administration of the active agents. Exemplary agents useful in the treatment or prevention of a neurodegenerative disease can include, but are not limited to, one or more of J147, DHA, fisetin, Aricept, and Dopa/Dopamine.

In some examples, treating a neurodegenerative disease includes one or more of increasing memory (such as an increase of at least 5%, at least 10%, at least 20%, or at least 50%, for example relative to no administration of the agent(s) that modulates (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels), decreasing memory loss (for example, decreases of at least 5%, at least 10%, at least 20%, or at least 50%, for example relative to no administration of the agent(s) that modulates (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels), and/or decreases neurodegenerative disease progression, such as the rate of such progression (for example, decreases of at least 5%, at least 10%, at least 20%, or at least 50%, for example relative to no administration of the agent(s) that modulates (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels). In some embodiments, the disclosed methods include measuring MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels.

In some examples, administration of the agent(s) that modulates (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels, treats or prevents a neurodegenerative disease, by decreasing the presence of MPTP and/or ATPsyn or their activity, such as a reduction of at least 5%, at least 10%, at least 20%, at least 50%, at least 75%, at least 80%, at least 90%, or at least 95%.

In some examples, administration of the agent(s) that modulates (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels, treats or prevents a neurodegenerative disease for example, by increasing AMPK activity in a subject. In some examples, the method includes increasing AMPK activity by at least 5% (such as at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 50%, at least 75%, at least 80%, at least 90%, at least 95%, at least 100%, at least 200%, at least 500%, or more) as compared with a control (such as no administration of the therapeutic agent). In some examples, administration of the agent(s) that modulates (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels, treats or prevents a neurodegenerative disease for example, by increasing ATP activity in a subject. In some examples, the method includes increasing ATP production by at least 5% (such as at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 50%, at least 75%, at least 80%, at least 90%, at least 95%, at least 100%, at least 200%, at least 500%, or more) as compared with a control (such as no administration of the therapeutic agent). In some examples, the method includes increasing NAD⁺ production by at least 5% (such as at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 50%, at least 75%, at least 80%, at least 90%, at least 95%, at least 100%, at least 200%, at least 500%, or more) as compared with a control (such as no administration of the therapeutic agent). In some examples, the method includes increasing NADH production by at least 5% (such as at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 50%, at least 75%, at least 80%, at least 90%, at least 95%, at least 100%, at least 200%, at least 500%, or more) as compared with a control (such as no administration of the therapeutic agent). In some examples, the method includes increasing NADPH production by at least 5% (such as at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 50%, at least 75%, at least 80%, at least 90%, at least 95%, at least 100%, at least 200%, at least 500%, or more) as compared with a control (such as no administration of the therapeutic agent). In some examples, the method includes decreasing MPTP (*e.g.*, amount or activity) by at least 5%

(such as at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 50%, at least 75%, at least 80%, at least 90%, at least 95%, or about at least 100%) as compared with a control (such as no administration of the therapeutic agent). In some examples, the method includes decreasing ATPase activity by at least 5% (such as at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 50%, at least 75%, at least 80%, at least 90%, at least 95%, or about at least 100%) as compared with a control (such as no administration of the therapeutic agent). In some examples, the method includes decreasing ATPsyn activity or expression by at least 5% (such as at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 50%, at least 75%, at least 80%, at least 90%, at least 95%, or about at least 100%) as compared with a control (such as no administration of the therapeutic agent). In some examples, the method includes decreasing mitochondrial calcium levels by at least 5% (such as at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 50%, at least 75%, at least 80%, at least 90%, at least 95%, or about at least 100%) as compared with a control (such as no administration of the therapeutic agent).

The method can include measuring memory levels, for example over a period of time (such as before and after administration of the therapeutic agent(s)). In particular examples, a change in memory or memory loss is determined relative to the memory of the subject at an earlier time (for example, prior to treatment with the agent(s) that modulates (*e.g.*, increase or decrease) MPTP (*e.g.*, amount or activity), ATPsyn activity or expression, ATPase activity, AMPK activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels).

Example 1

Materials and Methods

This examples provides materials and methods for the results described in Examples 2-7.

Study Design

These methods were used to investigate whether J147 protects SAMP8 mice from aging and AD-associated pathology and to assay the associated metabolic changes. Seventeen three-month old male SAMP8 mice were fed with control diet (LabDiet 5015, TestDiet, Richmond, IN) and eighteen three-month old male SAMP8 mice were fed with J147 diet (LabDiet 5015 + 200ppm J147, TestDiet) until they reached ten months old. At this age, SAMP8 mice present a strong phenotype [9]. The dose of J147 used was 200 ppm (~10mg/kg/day), which previously proved effective in AD transgenic mice [2, 3]. Fourteen three-month old male SAMP8 mice were used as

the young control group. The SAMP8 mice are an inbred strain and, as such, young SAMP8 mice were chosen as controls for young age. Given the seven month duration of the feeding paradigm, the effect of J147 diet could only be assessed in old SAMP8 mice, and any age-related changes defined by the comparison to the young SAMP8 animals. All mice were randomly assigned to experimental groups. The number of mice per group was determined based on previous experiments [9] and was sufficient to attain statistical power. Six old SAMP8 mice fed with control diet and two old SAMP8 mice fed with J147 diet died throughout the course of this study. Behavioral testing was carried out one month prior to sacrifice and collection of biological material. Data were analysed by blinded researchers when appropriate.

SAMP8 mice

The SAMP8 line was acquired from Harlan Laboratories (U.K.). Mouse body weights were measured regularly and no significant differences were found between the groups (FIG. 1).

Behavioral assays

Open field: The open field test was performed using MED Associates hardware and the Activity Monitor software according to the manufacturer's instructions (MED Associates Inc, St. Albans, VT, USA). Animals were individually placed into clear Plexiglas boxes (40.6 x 40.6 x 38.1 cm) surrounded by multiple bands of photo beams and optical sensors that measure horizontal and vertical activity. Their movement was detected as breaks within the beam matrices and automatically recorded for 30 minutes.

Elevated plus maze: The maze consisted of four arms (two open without walls and two enclosed by 15.25 cm high walls) 30 cm long and 5 cm wide in the shape of a plus. A video-tracking system (Noldus EthoVision) was used to automatically collect behavioral data. The software was installed on a PC computer with a digital video camera mounted overhead on the ceiling, which automatically detected and recorded when mice entered the open or closed arms of the maze and the time spent in each. Mice were habituated to the room 24 hr before testing and habituated to the maze for 1 min before testing by placing them in the center of the maze and blocking entry to the arms. Mice were then tested for a 5 min period and their behaviour recorded. Disinhibition was measured by comparing the time spent on the open arms to time spent on the closed arms.

Object recognition: Mice were tested in a standard home cage. Phase 1 (Habituation): Each mouse was placed into the apparatus (no objects present) for two 10 min sessions separated by 1-4 hours to habituate to testing environment. Phase 2 (Training): Two identical Velcro-backed

objects (object “A”) were attached into designated corners of the apparatus. The mouse was placed into the apparatus opposite to the objects and recorded by a camera for 10 min. Phase 3 (Test): One hour after training, the test phase began. Only one of the objects was replaced with a new object (object “B”). The mouse was placed into the apparatus opposite to the objects and recorded for 5 min. The apparatus was wiped and objects cleaned with 70% alcohol to remove odors between mice. “object recognition index” was calculated by dividing the amount of time spent with object B by the total time spent with objects A + B and multiplied by 100.

Barnes maze: The maze consisted of a flat circular surface (36” diameter) with 20 equally spaced holes (2” diameter) along the outer edge. One of the holes led to a dark hide box while the other 19 led to false boxes that were too small to be entered. The latency to enter the hide box was recorded. The test was conducted in three phases. Phase 1 (Training): A hide box was placed under one of the holes. Animals were placed into an opaque cylinder in the center of the maze for 30 sec to promote spatial disorientation at the start of the test. After 30 sec, the cylinder was removed and the animal explored the maze until it found and entered the hide box. The number of incorrect entries was scored. If the mouse failed to enter the box within 3 min, it was gently led into the box. The animal remained in the box for an additional 20 sec before it was removed from the box and gently placed into the home cage. Training is repeated three times a day for four days. The location of the hide box remained the same during every trial but it was shifted between subjects to reduce the potential for unintended intra-maze cues. Phase 2 (Retention): This phase measures retention of spatial memory following a delay. After a two day break from training, each animal was re-tested for a one day, three-trial session using the same hide box location as before. Phase 3 (Reversal): This phase examines memory reversal. On the day following the retention phase, a new hide box location was established 180 degrees to the original location. The same method as before was used and trials were repeated three times a day over two consecutive days.

Tissue preparation

Mice were anesthetized and their blood collected by cardiac puncture. After perfusing with PBS, their brains were removed. Half of the brain was fixed and processed for histology and the other half was dissected (to collect cortex and hippocampus) and prepared for Western blot (WB), RNA extraction, eicosanoid and metabolomic analysis.

Western blotting

Western blots were carried out as described previously [42]. The primary antibodies used were: 5-LOX (#610694, 1/1000) from BD Biosciences; eIF2 α (#2103, 1/5000), phospho-eIF2 α

Ser51 (#9721, 1/1000), HRP-conjugated rabbit anti-actin (#5125, 1/20,000), HSP40 (#4871, 1/1000), HSP60 (#4870, 1/5000), HSP70 (#4872, 1/5000), HSP90 (#4877, 1/5000), PSD-95 (#2507, 1/1000), SAPK/JNK (#9252, 1/1000), Phospho-SAPK/JNK Thr183/Tyr185 (#4668, 1/1000), phospho-tau Ser396 (#9632, 1/1000), from Cell Signaling Technology; tau (#A0024, 1/10000), from Dako Cytomation; Arc (#sc-15325, 1/2000), GFAP (#AB5804, 1/5000), SAP102 (#AB5170, 1/400), from Millipore; VCAM-1 (#sc-1504, 1/500) from Santa Cruz Biotechnology; APP C-Terminal (#A8717, 1/100000), from Sigma; SAP97 (#VAM-PS005, 1/1000), from Stressgen. Horseradish peroxidase-conjugated secondaries goat anti-rabbit, goat anti-mouse or rabbit anti-goat (Biorad) diluted 1/5000 were used. IgG heavy and light chains were detected by blotting the membrane directly with the mouse secondary antibody.

Immunohistochemistry

Immunohistochemistry was carried out as described previously [42]. Anti-Iba-1 (#019-19741, 1/4000, from Wako) and biotinylated rabbit secondary antibody (#BA1000, 1/400 from Vector Laboratories) were used. Number of microglia per mm² of hippocampus was quantified using the Image J software (NIH). Total counts in 2-4 sections per eight mouse brains of each group were determined in an unbiased fashion.

A β ELISA

A β 1-40 and 1-42 levels in hippocampal lysates were analyzed using the A β 1-40 and A β 1-42 ELISA kits from Invitrogen (# KMB3481 and # KMB3442, respectively).

Eicosanoid Analysis

Eicosanoids were prepared and analyzed as described previously [42].

Metabolomic analysis

Metabolomic analyses were conducted at Metabolon as described previously [43]. For statistical analyses and data display, any missing values were assumed to be below the limits of detection and imputed with the compound minimum (minimum value imputation). An estimate of the false discovery rate (Q-value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies, with Q<0.05 used as an indication of high confidence in a result.

RNA analysis

RNA was isolated from hippocampus using the RNeasy Plus Universal mini kit (Qiagen) and RNA analysis performed by Nanostring.

Nanostring: The nCounter GX Mouse Inflammation Kit (Nanostring, Seattle, USA) was used to measure a comprehensive set of 248 inflammation related mouse genes and six internal reference genes.

Whole transcriptome analysis: RNA-Seq libraries were prepared using the Illumina TruSeq Stranded mRNA Sample Prep Kit according to the manufacturer's instructions. Briefly, poly-A RNA was selected using poly dT-beads. mRNA was then fragmented and reverse transcribed. cDNA was end-repaired, adenylated and ligated with Illumina adapters with indexes. Adapter-ligated cDNA was then amplified. Libraries were pooled and sequenced single-end 50 base-pair (bp) on the Illumina HiSeq 2500 platform. Sequencing reads were mapped to the mm9 mouse genome using the spliced aligner STAR (2.3.0e) with default parameters [44]. Indices for the alignment were built using the Illumina iGenomes gene annotation for mm9 as a splice junction database and the sjdbOverhang parameter set to 100. Raw gene-level read counts were calculated using this same gene annotation with featureCounts (1.4.6) from the Subread package [45]. Expression normalization and differential analysis were carried out with DESeq2, using a false discovery rate cut-off of 0.1 [46]. The data discussed herein have been deposited in NCBI's Gene Expression Omnibus [47] and are accessible through GEO Series accession number GSE69244 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69244).

Bioinformatics and Statistics

Data in the figures is presented as group mean $\pm$ SD or as box-and-whisker plots indicating the group minimum, lower quartile, median, upper quartile, and group maximum. Data from the Western blotting and eicosanoids analyses were normalized to the average of the young SAMP8 control group. For metabolites, the measured values across the three groups were median-normalized to 1.

Data were analyzed and the functional/network analyses were generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA[®], QIAGEN Redwood City).

Metaboanalyst [48] was used to generate the heatmaps. Values were mean-centered and divided by the SD of each variable (scaled Z-score). Hierarchical clustering of RNA expression was performed using Euclidean distances and the Ward algorithm.

Statistical analysis of the three groups was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparison *post hoc* test was used. For data regarding multiple time

points, two-way repeated-measures ANOVA and post hoc Bonferroni corrected t tests were applied. GraphPad Prism 6 was used and exact *P* values are indicated (for *P* < 0.050). All data are mean ± SD.

Example 2

Behavioral Assessment

To address the effect of J147 on the SAMP8 phenotype, two groups of three-month old mice were fed with control or J147 diet for an additional seven months, while another group of three-month old mice was used as a young control group. The SAMP8 mice are an inbred strain and, as such, young SAMP8 mice were chosen as controls for young age. Given the seven-month duration of the feeding paradigm, the effect of the J147 diet could only be assessed in old SAMP8 mice, and age-related changes were defined by comparison to the young SAMP8 animals. At 10 months of age, SAMP8 mice present strong age- and AD-associated brain deterioration [6-9]. An in depth multiomics approach was used to integrate the physiological effects of J147 on both the aging and AD-associated phenotypes of these mice.

By monitoring the spontaneous behavior of mice in the open field assay, a decline in activity parameters was observed between the young and the old SAMP8 (FIGS. 2A-D). J147 had a positive effect on locomotor activity as it improved the average velocity and the number of vertical counts in the old SAMP8 mice. J147 had no effect on the body weights (FIG. 1).

To investigate whether J147 could prevent age-associated cognitive decline, mice were tested using the elevated plus maze (FIG. 2E), the object recognition test (FIG. 2F) and the Barnes maze (FIG. 2G). The elevated plus maze examines disinhibition behavior based on the aversion of normal mice to open spaces. Dementia is clinically associated with disinhibition and AD mouse models tend to exhibit increased disinhibition [3]. Accordingly, old SAMP8 mice spent significantly more time in the open arms compared to the young SAMP8 mice (FIG. 2E). However, this was not altered by J147 treatment.

The object recognition test evaluates recognition memory and is based on the spontaneous tendency of mice to spend more time exploring a novel object than a familiar one. The choice to explore the novel object reflects the use of learning and recognition memory. There was a significant decrease in the recognition index with age in SAMP8 mice, which was reversed by J147 (FIG. 2F).

The Barnes maze is used to analyze spatial learning and hippocampal-dependent memory. In this assay, mice use visual cues to locate a hidden box. With repeated trials, animals with an intact memory show a significant reduction in the time (latency) to locate the box. If the box is

moved to another location in the maze (reversal test), normal animals rapidly disengage from the previously learned information and re-learn the new location. No changes between the three groups were found in the escape latencies during the learning and the retention phases. However, when tested during the reversal phase, which is more sensitive to smaller deficits in memory and learning, differences were found in the capacity of mice to relearn the new location of the escape box (FIG. 2G). J147 significantly improved learning of the new location. These data show that J147 prevents the deterioration of several aspects of behavior and memory that are altered in old SAMP8 mice.

Example 3

Brain Hippocampal Protein Expression

Western blotting was used to investigate both protein alterations underlying the decline in cognitive performance of old SAMP8 mice and the therapeutic effects of J147. The expression of activity-regulated cytoskeleton-associated protein (Arc) and synapse-associated protein 102 (SAP102) decreased in old mice compared to young mice (FIGS. 3A and 3B), and treatment with J147 prevented these decreases.

It was then determined if these changes were accompanied by alterations in the levels of proteins involved in the cellular responses to stress relevant to aging and AD (FIGS. 3C-H). Phosphorylation of eukaryotic initiation factor 2 α (eIF2 α) occurs under a variety of stress conditions to control protein synthesis. Although total levels of eIF2 α were decreased in old SAMP8 mice compared to young controls, its phosphorylation was increased (FIGS. 3C and 3D), as has been reported in AD patients [10]. J147 reverted the changes in both the total levels and the phosphorylation of eIF2 α . Additionally, while the levels of heat shock protein 70 (HSP70) were not significantly altered between groups, changes in HSP40, HSP60 and HSP90 were detected in the old SAMP8 mice (FIGS. 3E-H). J147 restored HSP60 and HSP90 to levels similar to those found in the young control mice.

One hallmark of the AD brain is extracellular A β plaques. Although SAMP8 mice do not develop classical plaque pathology, they have a high content of A β and amyloid deposition around blood vessels [6, 9]. A β is the product of sequential cleavages of the amyloid precursor protein (APP). Processing of APP involves the formation of the C83 and C99 C-terminal fragments by α and β -secretases, respectively. Amyloidogenic processing of C99 by γ -secretase then generates A β [11]. Although no significant changes in the total levels of APP and the C99 and C83 fragments across the three groups were identified, there was a trend towards lower levels of APP and both the C99 and C83 fragments after treatment with J147 (FIGS. 4A and 4B). An increase in the level of

A β ₁₋₄₀ was detected in the hippocampus of old SAMP8 mice, which was significantly prevented by J147 (FIG. 4C). A β ₁₋₄₂ was below the limit of detection.

Tau pathology is another important feature of AD. Old SAMP8 mice showed increases in both tau protein and its phosphorylation at Ser396 (FIGS. 4D and 4E), an epitope affected in the human AD brain [12]. J147 prevented both of these alterations.

Example 4

Vascular Dysfunction and Inflammation

Given the relevance of inflammation in aging and AD, a detailed characterization of the inflammatory status of the aged SAMP8 brain and of the effects of J147 was carried out. AD is often accompanied by inflammation of the brain blood barrier (BBB), and the disruption of its permeability severely compromises neuronal homeostasis [13, 14]. A significant increase in the levels of vascular cell adhesion molecule 1 (VCAM-1), a protein associated with vascular endothelium inflammation, was detected in the hippocampus of old SAMP8 mice compared to the young SAMP8 controls (FIG. 5A). This increase was completely prevented by J147 treatment. In addition, old mice showed significantly higher levels of endogenous immunoglobulin G (IgG) (FIG. 5B), a consequence of disrupted BBB permeability [15], which was also prevented by J147. Together these results suggest that J147 helps to preserve BBB homeostasis and vascular function in aged SAMP8 mice.

Astrocytes are key constituents of the BBB, and astrocytic reactivity is increased in AD [16]. Previously, it was reported that an increased expression of glial fibrillary acidic protein (GFAP), a marker for astrocytes, was due to an increased number of astrocytes in the hippocampus of old SAMP8 mice [9]. The data herein confirmed an increase in GFAP levels from young to old SAMP8 mice and showed that J147 reduced GFAP expression (FIG. 5C). The number of microglia increased in the hippocampus of old mice compared to young (FIG. 5D), but J147 did not significantly alter their number.

Western blot analysis revealed an activation of the stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK), determined by its phosphorylation, in the hippocampus of old SAMP8 mice (FIGS. 5E and 5F). SAPK/JNK is activated in AD brains and may be the cause of abnormal tau phosphorylation [17]. J147 prevented the activation of SAPK/JNK in the old mice.

To expand upon these findings, the expression of a comprehensive panel of inflammatory genes was analyzed. The expression of a large number of genes was altered between young and old SAMP8 mice (50/248); most were upregulated (FIG. 5G). The vast majority of changes associated with J147 reverted alterations found in old SAMP8 mice toward expression levels in young mice.

These include mitogen-activated protein kinase (MAPK) kinases, such as Map3k7 and Map2k4, that are direct activators of SAPK/JNK, in accordance with the Western blotting data (FIGS. 5E and 5F).

J147 treatment was largely associated with an overall decrease in the expression of inflammatory markers in old mice, indicative of a reduction in stress-associated inflammation. However, the expression of some genes that are elevated in old mice, such as the components of the complement system C1qa, C1qb, C3 and C4a, were not altered by J147. Interestingly, there was a group of inflammation-associated genes whose expression was lowered in the old mice. Although most of these were not changed by J147, one that was restored by J147 is *Flt1* that encodes the vascular endothelial growth factor receptor 1 and may be related to the effect of J147 on the brain vasculature. These data show that J147 prevents a portion of the pro-inflammatory changes associated with aging in the SAMP8 mice.

Example 5

Eicosanoid Metabolism

To further elucidate the effects of J147 on inflammation, a detailed analysis of eicosanoid production in the brain cortex was conducted. Eicosanoids are a class of bioactive lipid mediators derived from the metabolism of polyunsaturated fatty acids (PUFAs) by cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome P450s as well as nonenzymatic pathways [18]. They are potent regulators of the inflammatory response in the periphery, but are much less studied in the brain.

Several fatty acids, including arachidonic acid (AA), docosahexaenoic acid (DHA), linoleic acid (LA) and adrenic acid, as well as their respective metabolites were analyzed (FIG. 6). J147 significantly increased the levels of DHA and restored those of adrenic acid. J147 also had a strong anti-oxidant effect, since most of the metabolites derived from the non-enzymatic oxidation of the different fatty acids were decreased in mice treated with J147 as compared to either young or old SAMP8 mice. These include the AA-metabolites 9-HETE and 8-iso-15-keto PGF₂ β ; the DHA-metabolites 11- and 13-HDoHEs; and the LA-metabolites 9-HODE and 13-HODE. Therefore, J147 can reduce the pro-oxidant status in the brain of old animals.

The levels of the cytochrome P450 metabolites 19-HETE and 20-HETE (AA derivatives), 19(20)-EpDPE (DHA derivative) and 9,10-DiHOME (LA derivative), which are known regulators of vascular dynamics [19, 20], were altered with J147 treatment. 20-HETE and 9,10-DiHOME were also altered by aging and this was prevented by J147. In addition, several COX metabolites were changed. TXB₂, a product of TXA₂, was increased in old SAMP8 mice and lowered by

J147. The thromboxane pathway is implicated in platelet aggregation, adhesion and vascular contraction during inflammation [21]. Thus, these data further indicate that J147 reduces the decline in brain vascular health that occurs during aging.

5

Example 6

Small Molecule Metabolism

To address the therapeutic effects of J147 on brain and whole body health, a global metabolic profiling study was performed with blood plasma and brain cortical tissue. 195 of 593 (32.9%) and 105 of 493 (21.3%) assayed biochemicals differed significantly in the plasma and cortex, respectively, between the three groups. The heatmaps in FIGS. 7A and 7B depict the major altered biological pathways.

Global pathway changes regarding the metabolism of amino acids, peptides and lipids were detected in the plasma (FIG. 7A). In the brain, significant changes were associated with amino acid and lipid metabolism and, importantly, neurotransmission and energy production (FIG. 7B).

Venn diagrams highlighting the significant changes in plasma and cortex that differentiate the comparisons between the young, old and old+J147 groups are shown in FIGS. 7C and 7D. Fold changes of the overlapping metabolites were correlated between the two comparisons (FIGS. 7E and 7F). Treatment with J147 rescued changes in all of the 31 plasma metabolites also found altered in old SAMP8 mice. This accounts for 76% of all differences between old SAMP8 treated with J147 and old SAMP8 fed with control diet. In the cortex, J147 rescued changes in 11 biochemicals (out of 12), representing 55% of all differences between J147 treated and untreated old SAMP8 mice.

FIG. 7G shows the specific biological groups of metabolites found affected in the plasma organized by descending number of metabolites changed (for all biochemicals see Table 1).

Numerous γ -glutamyl amino acids, branched-chain amino acids (BCAAs) and dipeptides were downregulated with age, while several acylcarnitines and PUFAs were highly elevated in the old SAMP8 mice. In both cases, J147 reversed most of these changes towards the younger phenotype.

Table 1: List of 195 (out of 593) biochemicals found significantly altered in the blood plasma of young SAMP8, old SAMP8 and old SAMP8 mice fed J147. Fold changes and specific *P* values are indicated. One-way ANOVA followed by Tukey-Kramer post-hoc test (*n* = 5/group).

			Old/Young		J147/Old	
BIOCHEMICAL	SUPER PATHWAY	SUB PATHWAY	Fold change	p value	Fold change	p value

asparagine	Amino Acid	Alanine and Aspartate Metabolism	0.661	< 0.001	1.213	
N-acetylalanine	Amino Acid	Alanine and Aspartate Metabolism	0.713	0.005	0.926	
N-acetyl asparagine	Amino Acid	Alanine and Aspartate Metabolism	0.482	0.025	1.524	
creatinine	Amino Acid	Creatine Metabolism	0.681	0.039	1.024	
N-acetylthreonine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.561	0.015	0.731	
dimethylglycine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.674	0.020	1.233	
threonine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.728	0.023	1.186	
glycine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.600		0.723	
4-guanidinobutanoate	Amino Acid	Guanidino and Acetamido Metabolism	0.401	0.027	1.620	
1-methylimidazoleacetate	Amino Acid	Histidine Metabolism	2.376	0.017	0.703	
4-imidazoleacetate	Amino Acid	Histidine Metabolism	1.396		1.728	
N-acetyl-3-methylhistidine	Amino Acid	Histidine Metabolism	1.887		0.354	0.043
N-acetylvaline	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.509	< 0.001	1.008	
N-acetyl leucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.370	< 0.001	1.239	
beta-hydroxyisovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.443	< 0.001	1.187	
3-hydroxyisobutyrate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.483	0.001	1.387	
4-methyl-2-oxopentanoate	Amino Acid	Leucine, Isoleucine and	0.579	0.001	1.041	

		Valine Metabolism				
tiglyl carnitine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.490	0.002	1.544	
isovalerylcarnitine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.570	0.010	1.305	
ethylmalonate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	1.895	0.022	0.594	0.048
isovalerylglycine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.543	0.040	1.388	
isoleucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.594	< 0.001	1.209	
leucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.594	< 0.001	1.156	
N-acetyl isoleucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.373	< 0.001	1.350	
valine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.616	< 0.001	1.288	0.016
tigloylglycine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.329	< 0.001	0.895	
alpha-hydroxyisocaproate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.693		0.781	
3-methyl-2-oxovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.733		0.927	
N6-acetyllysine	Amino Acid	Lysine Metabolism	0.689	0.001	0.991	
N2,N6-diacetyllysine	Amino Acid	Lysine Metabolism	0.625	0.002	1.252	
2-aminoadipate	Amino Acid	Lysine	0.522	0.003	1.066	

		Metabolism				
N2-acetyllysine	Amino Acid	Lysine Metabolism	0.634	0.004	1.015	
glutarate (pentanedioate)	Amino Acid	Lysine Metabolism	0.276	0.013	1.404	
2-oxoadipate	Amino Acid	Lysine Metabolism	0.675	0.034	0.970	
pipecolate	Amino Acid	Lysine Metabolism	0.598	< 0.001	0.815	
2-hydroxybutyrate (AHB)	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.494	< 0.001	1.285	
N-acetylmethionine sulfoxide	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.413	0.003	1.417	
2-aminobutyrate	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.641	0.004	1.362	0.049
N-acetylmethionine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.532	0.013	1.284	
methionine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	1.640	0.015	1.045	
S-methylcysteine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	1.433		1.353	
N-acetyltaurine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.773		0.786	
N-formylmethionine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	1.492		1.189	0.037
phenylpyruvate	Amino Acid	Phenylalanine and Tyrosine Metabolism	0.555	0.001	1.090	
4-hydroxycinnamate	Amino Acid	Phenylalanine and Tyrosine Metabolism	0.248	0.004	3.555	0.013
phenylalanine	Amino Acid	Phenylalanine and Tyrosine Metabolism	0.808	0.007	1.068	

4-hydroxyphenylpyruvate	Amino Acid	Phenylalanine and Tyrosine Metabolism	0.257	0.014	0.949	
tyrosine	Amino Acid	Phenylalanine and Tyrosine Metabolism	0.666	0.016	1.134	
4-hydroxyphenylacetate	Amino Acid	Phenylalanine and Tyrosine Metabolism	0.293	0.017	1.875	
4-hydroxyphenylacetyl glycine	Amino Acid	Phenylalanine and Tyrosine Metabolism	0.426	0.020	1.399	
N-acetyltyrosine	Amino Acid	Phenylalanine and Tyrosine Metabolism	0.523	0.034	0.845	
N-acetylphenylalanine	Amino Acid	Phenylalanine and Tyrosine Metabolism	0.877		0.709	
3-(4-hydroxyphenyl)lactate	Amino Acid	Phenylalanine and Tyrosine Metabolism	0.659		0.818	
indole-3-carboxylic acid	Amino Acid	Tryptophan Metabolism	0.357	0.001	0.756	
kynurenate	Amino Acid	Tryptophan Metabolism	0.343	0.003	1.089	
xanthurenate	Amino Acid	Tryptophan Metabolism	0.294	0.005	1.982	
picolinate	Amino Acid	Tryptophan Metabolism	0.583	0.008	1.486	
N-acetyltryptophan	Amino Acid	Tryptophan Metabolism	0.682	0.026	0.912	
anthranilate	Amino Acid	Tryptophan Metabolism	0.646	0.027	1.019	
indoleacetate	Amino Acid	Tryptophan Metabolism	0.528	0.030	1.571	
N-acetylkynurenine (2)	Amino Acid	Tryptophan Metabolism	0.868		0.833	
homocitrulline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.427	0.001	1.218	
urea	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.682	0.002	1.145	
ornithine	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.666	0.002	1.070	
N-acetylarginine	Amino Acid	Urea cycle;	0.498	0.004	1.625	0.049

		Arginine and Proline Metabolism				
pro-hydroxy-pro	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.440	0.004	0.929	
trans-4-hydroxyproline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.349	< 0.001	1.248	
cyclo(L-phe-L-pro)	Peptide	Dipeptide	0.366	0.001	1.898	0.045
glycylvaline	Peptide	Dipeptide	0.415	0.001	1.127	
cyclo(gly-pro)	Peptide	Dipeptide	0.316	0.001	2.244	0.048
pyroglutamylvaline	Peptide	Dipeptide	0.195	0.002	2.076	
isoleucylglycine	Peptide	Dipeptide	0.401	0.003	1.163	
glycylproline	Peptide	Dipeptide	0.276	0.005	1.878	
cyclo(leu-pro)	Peptide	Dipeptide	0.408	0.009	2.284	0.019
isoleucylaspartate	Peptide	Dipeptide	0.422	0.012	1.553	
cis-Cyclo[L-ala-L-Pro]	Peptide	Dipeptide	0.501	0.013	2.099	0.007
glycylleucine	Peptide	Dipeptide	0.442	0.032	1.318	
carnosine	Peptide	Dipeptide Derivative	0.389	0.006	1.251	
gamma-glutamylphenylalanine	Peptide	Gamma-glutamyl Amino Acid	0.481	0.004	1.813	0.024
gamma-glutamylthreonine	Peptide	Gamma-glutamyl Amino Acid	0.515	0.011	1.763	0.035
gamma-glutamylvaline	Peptide	Gamma-glutamyl Amino Acid	0.536	0.011	1.537	
gamma-glutamylleucine	Peptide	Gamma-glutamyl Amino Acid	0.531	0.021	1.398	
gamma-glutamyltryptophan	Peptide	Gamma-glutamyl Amino Acid	0.497	0.022	1.720	
gamma-glutamylalanine	Peptide	Gamma-glutamyl Amino Acid	0.631	0.028	1.092	
gamma-glutamylisoleucine	Peptide	Gamma-glutamyl Amino Acid	0.602	0.030	1.309	
gamma-glutamyl-2-aminobutyrate	Peptide	Gamma-glutamyl Amino Acid	0.287	0.039	3.008	
gamma-glutamyltyrosine	Peptide	Gamma-glutamyl Amino Acid	0.599	0.046	1.293	
erythronate	Carbohydrate	Aminosugar Metabolism	0.746	0.039	0.979	
galactonate	Carbohydrate	Fructose, Mannose and Galactose Metabolism	0.548	0.001	1.233	
1,5-anhydroglucitol (1,5-AG)	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate	0.471	< 0.001	0.987	

		Metabolism				
arabitol	Carbohydrate	Pentose Metabolism	0.548	< 0.001	1.587	0.004
threitol	Carbohydrate	Pentose Metabolism	0.596	0.014	1.469	
xylonate	Carbohydrate	Pentose Metabolism	0.642	0.017	0.926	
arabinose	Carbohydrate	Pentose Metabolism	1.144		0.657	0.034
tricarballoylate	Energy	TCA Cycle	0.153	0.007	1.872	
fumarate	Energy	TCA Cycle	0.487	0.024	1.260	
malate	Energy	TCA Cycle	0.521	0.036	1.231	
alpha-ketoglutarate	Energy	TCA Cycle	0.633	0.043	1.251	
N-oleoyltaurine	Lipid	Endocannabinoid	3.617	0.004	0.381	0.010
oleic ethanolamide	Lipid	Endocannabinoid	2.759	0.027	0.561	
propionylglycine	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	0.497	0.022	1.484	
decanoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	3.150	< 0.001	0.571	0.006
myristoleoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	2.880	0.001	0.522	0.007
laurylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	2.318	0.003	0.578	0.019
oleoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	2.192	0.005	0.633	0.047
myristoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	2.124	0.017	0.635	
heptanoyl glycine	Lipid	Fatty Acid Metabolism(Acyl Glycine)	2.244	0.027	0.964	
hexanoylglycine	Lipid	Fatty Acid Metabolism(Acyl Glycine)	2.008	0.044	0.338	0.010
malonate (propanedioate)	Lipid	Fatty Acid Synthesis	0.556	0.003	1.129	
2-aminooctanoate	Lipid	Fatty Acid, Amino	1.660	0.016	0.826	
15-methylpalmitate (isobar with 2-methylpalmitate)	Lipid	Fatty Acid, Branched	1.623		1.010	
hexadecanedioate	Lipid	Fatty Acid, Dicarboxylate	1.531	0.004	0.759	0.039
2-hydroxyadipate	Lipid	Fatty Acid, Dicarboxylate	0.376	0.005	1.826	

undecanedioate	Lipid	Fatty Acid, Dicarboxylate	2.514	0.006	0.930	
azelate (nonanedioate)	Lipid	Fatty Acid, Dicarboxylate	2.410	0.016	0.836	
tetradecanedioate	Lipid	Fatty Acid, Dicarboxylate	1.413	0.024	0.880	
dimethylmalonic acid	Lipid	Fatty Acid, Dicarboxylate	0.669	0.050	1.612	0.023
2-hydroxypalmitate	Lipid	Fatty Acid, Monohydroxy	1.405	0.032	0.707	0.029
chiro-inositol	Lipid	Inositol Metabolism	0.419	0.001	1.585	
myo-inositol	Lipid	Inositol Metabolism	0.700	0.048	1.153	
eicosenoate (20:1n9 or 11)	Lipid	Long Chain Fatty Acid	2.826	0.004	0.512	0.022
margarate (17:0)	Lipid	Long Chain Fatty Acid	1.765	0.013	0.785	
stearate (18:0)	Lipid	Long Chain Fatty Acid	1.388	0.044	0.917	
1- pentadecanoylglycerophosphocholine (15:0)	Lipid	Lysolipid	0.618	0.001	1.107	
1- eicosapentaenoylglycerophosphocholine (20:5n3)	Lipid	Lysolipid	0.221	0.004	0.950	
1- docosapentaenoylglycerophosphocholine (22:5n6)	Lipid	Lysolipid	4.360	0.004	0.821	
oleoyl-linoleoyl- glycerophosphocholine (2)	Lipid	Lysolipid	1.365	0.006	1.039	
stearoyl-arachidonoyl- glycerophosphocholine (2)	Lipid	Lysolipid	1.642	0.009	0.931	
1-arachidonoylglycerophosphate	Lipid	Lysolipid	2.647	0.011	0.565	
stearoyl-arachidonoyl- glycerophosphocholine (1)	Lipid	Lysolipid	1.836	0.021	1.030	
1- arachidonoylglycerophosphoinositol	Lipid	Lysolipid	1.948	0.027	0.793	
2-oleoylglycerophosphocholine	Lipid	Lysolipid	1.627	0.028	0.825	
1- arachidonoylglycerophosphocholine (20:4n6)	Lipid	Lysolipid	1.417	0.037	1.023	
palmitoyl-arachidonoyl- glycerophosphocholine (1)	Lipid	Lysolipid	1.421	0.038	1.166	
stearoyl-linoleoyl- glycerophosphocholine (2)	Lipid	Lysolipid	1.275	0.044	1.090	
palmitoyl-palmitoyl- glycerophosphocholine (1)	Lipid	Lysolipid	1.332		1.505	
1-	Lipid	Lysolipid	0.618		0.895	

docosapentaenylglycerophosphocholine (22:5n3)						
palmitoyl-arachidonoyl-glycerophosphocholine (2)	Lipid	Lysolipid	1.244		1.090	
palmitoyl-oleoyl-glycerophosphocholine (1)	Lipid	Lysolipid	1.384		1.022	
1-eicosatrienylglycerophosphoethanolamine	Lipid	Lysolipid	2.514		0.253	0.024
pelargonate (9:0)	Lipid	Medium Chain Fatty Acid	1.445	0.033	0.774	
3-hydroxy-3-methylglutarate	Lipid	Mevalonate Metabolism	0.587	0.018	1.465	
1-docosaheptaenylglycerol	Lipid	Monoacylglycerol	0.619		0.543	
glycerophosphorylcholine (GPC)	Lipid	Phospholipid Metabolism	1.760	0.002	0.733	0.040
choline	Lipid	Phospholipid Metabolism	0.887		0.890	
ethanolamine	Lipid	Phospholipid Metabolism	0.698		0.373	
eicosapentaenoate (EPA; 20:5n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.303	0.001	0.823	
mead acid (20:3n9)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	4.432	0.002	0.618	
dihomo-linoleate (20:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	4.007	0.004	0.460	0.030
dihomo-linolenate (20:3n3 or n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	1.985	0.005	0.630	0.029
docosatrienoate (22:3n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	2.643	0.020	0.318	0.012
adrenate (22:4n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	3.410	0.036	0.698	
arachidonate (20:4n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	1.665	0.046	0.931	
docosadienoate (22:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	1.798	0.049	0.569	
docosapentaenoate (n6 DPA; 22:5n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	5.110	< 0.001	0.463	0.002
inosine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/I	5.644		0.019	0.041

		nosine containing				
N6-carbamoylthreonyladenosine	Nucleotide	Purine Metabolism, Adenine containing	1.627	0.037	0.581	0.024
N1-methylguanosine	Nucleotide	Purine Metabolism, Guanine containing	1.402	0.016	0.664	0.006
N2,N2-dimethylguanosine	Nucleotide	Purine Metabolism, Guanine containing	1.656	0.046	0.741	
guanosine	Nucleotide	Purine Metabolism, Guanine containing	2.306		0.422	0.048
3-ureidopropionate	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.585	0.005	1.200	
5-methyluridine (ribothymidine)	Nucleotide	Pyrimidine Metabolism, Uracil containing	2.153	0.031	0.722	
beta-alanine	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.596		0.641	
glucarate (saccharate)	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	0.615	0.014	1.424	
gulonic acid	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	0.520	0.034	1.521	
biliverdin	Cofactors and Vitamins	Hemoglobin and Porphyrin Metabolism	1.803		0.419	0.015
nicotinamide N-oxide	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	2.599	0.030	0.699	
1-methylnicotinamide	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	2.835	0.047	0.354	0.047
riboflavin (Vitamin B2)	Cofactors and Vitamins	Riboflavin Metabolism	0.589	0.003	1.347	
flavin adenine dinucleotide (FAD)	Cofactors and Vitamins	Riboflavin Metabolism	0.834	0.042	1.016	
dihydrobiopterin	Cofactors and Vitamins	Tetrahydrobiopterin Metabolism	0.750		0.673	
4-sulfooxy-methylbenzoate	Xenobiotics	Benzoate Metabolism	0.158	0.010	4.081	
4-hydroxyhippurate	Xenobiotics	Benzoate	0.359	0.016	1.519	

		Metabolism				
3-methoxycatechol sulfate (2)	Xenobiotics	Benzoate Metabolism	0.445	0.026	0.804	
p-hydroxybenzaldehyde	Xenobiotics	Benzoate Metabolism	0.668	0.042	1.115	
2-hydroxyhippurate (salicylurate)	Xenobiotics	Benzoate Metabolism	0.056	< 0.001	2.100	
3-methyl catechol sulfate (1)	Xenobiotics	Benzoate Metabolism	0.075	< 0.001	0.764	
S-(3-hydroxypropyl)mercapturic acid (HPMA)	Xenobiotics	Chemical	0.354	< 0.001	1.285	
3-hydroxypyridine sulfate	Xenobiotics	Chemical	0.094	0.001	0.766	
2-oxo-1-pyrrolidinepropionate	Xenobiotics	Chemical	0.366	0.001	1.081	
2-hydroxyisobutyrate	Xenobiotics	Chemical	1.507	0.034	0.974	
N-methylpipercolate	Xenobiotics	Chemical	0.230	< 0.001	1.820	0.032
4-hydroxychlorothalonil	Xenobiotics	Chemical	0.792		2.298	< 0.001
glycolate (hydroxyacetate)	Xenobiotics	Chemical	0.862		0.992	0.013
6-oxopiperidine-2-carboxylic acid	Xenobiotics	Drug	0.563	0.001	1.353	
4-acetylphenol sulfate	Xenobiotics	Drug	0.355	0.006	1.420	
salicylate	Xenobiotics	Drug	0.079	< 0.001	2.089	
2-piperidinone	Xenobiotics	Food Component/ Plant	0.475	0.015	1.621	
N-(2-furoyl)glycine	Xenobiotics	Food Component/ Plant	0.248	0.037	1.662	
daidzein	Xenobiotics	Food Component/ Plant	0.363	0.043	2.614	
homostachydrine	Xenobiotics	Food Component/ Plant	0.083	< 0.001	1.172	
methyl glucopyranoside (alpha + beta)	Xenobiotics	Food Component/ Plant	0.019	< 0.001	1.255	
quininate	Xenobiotics	Food Component/ Plant	0.029	< 0.001	1.987	
stachydrine	Xenobiotics	Food Component/ Plant	0.095	< 0.001	1.378	
2,3-dihydroxyisovalerate	Xenobiotics	Food Component/ Plant	0.522		4.385	< 0.001

To investigate which biological pathways and diseases/functions could be associated with these alterations, Ingenuity Pathways Analysis (IPA) was carried out with biochemicals present in the Human Metabolome Database (HMDB). Many of the metabolites identified in FIG. 7 do not have an established biological pathway in HMDB. The top canonical pathways altered in the plasma are shown in FIGS. 8A and 8B, and confirm changes in amino acid, protein metabolism, urea cycle and mitochondrial energetics (tricarboxylic acid cycle (TCA) and oxidative phosphorylation) found between the young and old SAMP8 mice. Some of the diseases and functions predicted to be associated with these changes relevant to aging include cancer,

gastrointestinal and hepatic dysfunction, endocrine system, energy metabolism and inflammatory responses (FIG. 8C). Most of these were also found associated with the metabolites changed by J147, indicative that J147 might be rescuing certain aspects of these diseases and functions that are changed with age.

Although the total number of metabolites altered in the brain of old SAMP8 mice was lower than in the plasma, the associations determined by IPA were strong (FIGS. 8D and 8E). Some of the metabolites of interest participate in energy production processes, amino acid metabolism, G protein-coupled receptor (GPCR) and cAMP signaling, neuronal homeostasis and lipid metabolism (FIG. 7J and 7K) (for all biochemicals see Table 2). Specifically, alterations in glycolytic and TCA intermediates in old SAMP8 mice (FIG. 7G) are indicative of mitochondrial dysfunction, which is characteristic of aging and AD [22]. J147 also preserved the levels of glutamate, the principal neurotransmitter in the brain and a product of the TCA intermediate α -ketoglutarate, which was also rescued by J147 (FIG. 7G). The levels of cAMP were elevated in old SAMP8 mice and were lowered by J147. cAMP is an intracellular signal transduction molecule crucial for many biological processes and its upregulation has been associated with AD [23]. The predicted diseases and functions are consistent with these alterations and include cancer, stress pathways, cellular survival/growth and maintenance, neurological disease and energy metabolism (FIG. 8F).

Table 2: List of 105 (out of 493) biochemicals found significantly altered in the brain cortex of young SAMP8, old SAMP8 and old SAMP8 mice fed J147. Fold changes and specific *P* values are indicated. One-way ANOVA followed by Tukey-Kramer post-hoc test (*n* = 5/group).

BIOCHEMICAL	SUPER PATHWAY	SUB PATHWAY	Old / Young		J147 / Old	
			Fold change	p value	Fold change	p value
asparagine	Amino Acid	Alanine and Aspartate Metabolism	0.840	0.026	1.110	
glutamate	Amino Acid	Glutamate Metabolism	0.862	0.001	1.105	0.015
pyroglutamine	Amino Acid	Glutamate Metabolism	1.199	0.002	1.051	
glutamine	Amino Acid	Glutamate Metabolism	1.188	0.008	0.955	
N-acetylglutamate	Amino Acid	Glutamate Metabolism	0.855	0.009	1.084	
N-acetyl-aspartyl-glutamate (NAAG)	Amino Acid	Glutamate Metabolism	0.762	0.021	1.040	
glutathione, oxidized (GSSG)	Amino Acid	Glutathione Metabolism	0.859	0.019	0.973	

cysteine-glutathione disulfide	Amino Acid	Glutathione Metabolism	0.932		0.704	
N-acetylserine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.856	< 0.001	0.920	
serine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.777	0.010	0.998	
threonine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.838	0.017	1.064	
allo-threonine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.803	0.043	0.976	
histidine	Amino Acid	Histidine Metabolism	1.364	0.001	0.945	
trans-urocanate	Amino Acid	Histidine Metabolism	2.075	0.005	0.676	
3-hydroxyisobutyrate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.722	0.001	1.036	
ethylmalonate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	1.336	0.025	0.768	0.038
methylsuccinate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	1.119	0.027	0.890	0.023
N-acetylleucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.838		0.918	0.019
saccharopine	Amino Acid	Lysine Metabolism	0.574	0.001	1.362	
pipecolate	Amino Acid	Lysine Metabolism	0.784	0.030	0.888	
2-hydroxybutyrate (AHB)	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.603	< 0.001	1.372	0.013
methionine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	1.871	0.001	0.798	
S-adenosylmethionine (SAM)	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.820	0.013	1.148	

S-methylcysteine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	1.931	0.023	0.866	
N-acetyltaurine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.829	0.028	0.920	
3-[3-(sulfooxy)phenyl]propanoic acid	Amino Acid	Phenylalanine and Tyrosine Metabolism	10.441	0.006	0.404	
phenylalanine	Amino Acid	Phenylalanine and Tyrosine Metabolism	1.163	0.031	0.924	
N-acetyltyrosine	Amino Acid	Phenylalanine and Tyrosine Metabolism	0.885		0.735	
indoleacetate	Amino Acid	Tryptophan Metabolism	3.847	0.006	0.696	
pro-hydroxy-pro	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.489	< 0.001	1.068	
ornithine	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.496	0.010	0.715	
trans-4-hydroxyproline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.239	< 0.001	1.536	
homocitrulline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.495	0.002	0.975	0.003
citrulline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	1.168		1.379	0.048
alanylanine	Peptide	Dipeptide	0.440	0.040	0.849	
pyroglutamylvaline	Peptide	Dipeptide	1.199		0.521	0.024
homocarnosine	Peptide	Dipeptide Derivative	1.676	0.007	0.877	
gamma-glutamylglutamine	Peptide	Gamma-glutamyl Amino Acid	1.443	0.002	0.950	
gamma-glutamylmethionine	Peptide	Gamma-glutamyl Amino Acid	2.292	0.002	0.762	
gamma-glutamylphenylalanine	Peptide	Gamma-glutamyl Amino Acid	1.442	0.005	1.006	
N-acetylneuraminate	Carbohydrate	Aminosugar	0.887	0.006	0.983	

		Metabolism				
erythronate	Carbohydrate	Aminosugar Metabolism	0.915	0.032	1.034	
N-acetylglucosamine 6-phosphate	Carbohydrate	Aminosugar Metabolism	0.906		0.965	
pyruvate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.671	0.001	1.083	
3-phosphoglycerate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1.741	0.016	0.732	
phosphoenolpyruvate (PEP)	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1.661	0.020	0.804	
2-phosphoglycerate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1.568	0.049	0.866	
1,5-anhydroglucitol (1,5-AG)	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.471	< 0.001	0.876	
UDP-glucuronate	Carbohydrate	Nucleotide Sugar	0.819	0.007	1.030	
UDP-N-acetylglucosamine	Carbohydrate	Nucleotide Sugar	0.848	0.008	1.011	
cytidine 5'-monophospho-N-acetylneuraminic acid	Carbohydrate	Nucleotide Sugar	0.892	0.044	1.015	
arabinose	Carbohydrate	Pentose Metabolism	1.432	0.001	0.913	
ribitol	Carbohydrate	Pentose Metabolism	0.625	< 0.001	1.319	0.012
xylitol	Carbohydrate	Pentose Metabolism	0.651		0.871	
arabonate/xylonate	Carbohydrate	Pentose Phosphate Pathway	1.206	0.015	0.899	
sedoheptulose-7-phosphate	Carbohydrate	Pentose Phosphate Pathway	0.782	0.032	1.056	
ribose 1-phosphate	Carbohydrate	Pentose Phosphate Pathway	1.004		0.840	0.045
alpha-ketoglutarate	Energy	TCA Cycle	0.895	0.019	1.109	0.028
succinylcarnitine	Energy	TCA Cycle	1.362	0.001	0.829	0.021
citrate	Energy	TCA Cycle	1.262	0.033	1.181	
isocitrate	Energy	TCA Cycle	1.637	0.037	1.049	
deoxycarnitine	Lipid	Carnitine Metabolism	0.914		0.967	
acetyl CoA	Lipid	Fatty Acid Metabolism	1.608	0.008	0.930	
propionylglycine	Lipid	Fatty Acid	0.589		0.880	

		Metabolism (also BCAA Metabolism)				
acetylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	1.225	0.016	0.910	
malonylcarnitine	Lipid	Fatty Acid Synthesis	1.134		1.285	0.018
2-methylmalonyl carnitine	Lipid	Fatty Acid Synthesis	0.847		0.798	
2-hydroxyadipate	Lipid	Fatty Acid, Dicarboxylate	0.579	0.001	1.137	
2-hydroxyglutarate	Lipid	Fatty Acid, Dicarboxylate	0.838	0.016	0.980	
2-hydroxypalmitate	Lipid	Fatty Acid, Monohydroxy	1.839	0.001	1.028	
3-hydroxylaurate	Lipid	Fatty Acid, Monohydroxy	1.439	0.019	0.821	
2-hydroxystearate	Lipid	Fatty Acid, Monohydroxy	1.725	< 0.001	1.117	
scyllo-inositol	Lipid	Inositol Metabolism	0.783		0.923	
palmitoyl-oleoyl- glycerophosphoglycerol (2)	Lipid	Lysolipid	0.934	0.019	0.983	
stearoyl-arachidonoyl- glycerophosphoserine (1)	Lipid	Lysolipid	1.186		1.047	
3-hydroxy-3-methylglutarate	Lipid	Mevalonate Metabolism	0.824	< 0.001	1.002	
1-stearoylglycerol (1- monostearin)	Lipid	Monoacylglycerol	0.733	0.007	1.085	
ethanolamine	Lipid	Phospholipid Metabolism	0.841	0.016	1.046	
cytidine 5'-diphosphocholine	Lipid	Phospholipid Metabolism	0.856	0.029	0.991	
cytidine-5'- diphosphoethanolamine	Lipid	Phospholipid Metabolism	0.899	0.030	1.035	
choline phosphate	Lipid	Phospholipid Metabolism	0.954		0.946	
docosapentaenoate (n3 DPA; 22:5n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.592	< 0.001	1.085	
eicosapentaenoate (EPA; 20:5n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.425	0.005	0.672	
dihomo-linolenate (20:3n3 or n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.636	< 0.0001	1.277	0.004
docosapentaenoate (n6 DPA; 22:5n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	1.916	0.034	1.157	

docosatrienoate (22:3n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.519	0.037	1.180	
cholestanol	Lipid	Sterol	1.510	0.025	0.907	
urate	Nucleotide	Purine Metabolism, (Hypo)Xanthine/ Inosine containing	1.382	0.017	0.859	
adenosine 3',5'-cyclic monophosphate (cAMP)	Nucleotide	Purine Metabolism, Adenine containing	1.218	0.031	0.813	0.024
adenylosuccinate	Nucleotide	Purine Metabolism, Adenine containing	1.101		0.166	0.024
adenosine 5'-monophosphate (AMP)	Nucleotide	Purine Metabolism, Adenine containing	1.122		0.617	0.043
orotidine	Nucleotide	Pyrimidine Metabolism, Orotate containing	0.894	0.035	1.042	
N-acetyl-beta-alanine	Nucleotide	Pyrimidine Metabolism, Uracil containing	1.129		0.698	0.016
ascorbate (Vitamin C)	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	1.198		1.030	
nicotinamide ribonucleotide (NMN)	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	1.269		1.062	
pantothenate	Cofactors and Vitamins	Pantothenate and CoA Metabolism	1.735	0.001	0.828	
3'-dephosphocoenzyme A	Cofactors and Vitamins	Pantothenate and CoA Metabolism	1.419	0.034	1.097	
phosphopantetheine	Cofactors and Vitamins	Pantothenate and CoA Metabolism	1.557	0.036	1.203	
dihydrobiopterin	Cofactors and Vitamins	Tetrahydrobiopterin Metabolism	0.678		0.904	
salicylate	Xenobiotics	Drug	0.604	0.034	1.081	
ergothioneine	Xenobiotics	Food Component/Plant	2.109	< 0.001	0.631	0.003
2,3-dihydroxyisovalerate	Xenobiotics	Food Component/Plant	2.057	0.023	0.510	0.029
methyl glucopyranoside (alpha + beta)	Xenobiotics	Food Component/Plant	0.021	< 0.001	1.552	
stachydrine	Xenobiotics	Food Component/Plant	0.165	< 0.001	0.884	
homocitrate	Xenobiotics	Food	0.901		1.226	0.019

		Component/Plant				
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Example 7

Whole transcriptome

To elucidate how changes in behavior, proteomics and metabolomics are related to alterations in gene expression, whole transcriptome analysis was carried out with hippocampal tissue. 5279 genes were altered between the old and young SAMP8 mice and 150 genes were changed with J147 treatment (FIG. 9A). The heatmap in FIG. 9B shows a rescuing effect by J147 of changes verified between the young and old SAMP8 mice. Correlation of the expression of the overlapping 121 genes between the two comparisons confirms that most of these (116 genes; 77% of total genes changed with J147) are associated with a rescue of age-related changes in gene expression (FIG. 9C).

The IPA of canonical pathways revealed a number of important signaling pathways related to brain function, including axonal guidance, G-protein coupled receptor (GPCR), protein kinase A (PKA), cAMP and neuronal cAMP response element-binding protein (CREB) signaling (FIG. 9D). Importantly, treatment with J147 altered the expression of genes associated with some of these pathways, namely cAMP, GPCR and CREB signaling. Also of interest are the changes in the renin-angiotensin and endothelin-1 signaling in old SAMP8 mice, which are important regulators of vascular function, supporting the Western blotting data regarding alterations in the brain vasculature (FIGS. 5A and 5B).

The large dataset obtained with the whole transcriptome allowed for a more informative prediction on the diseases and functions associated with aging. These included cancer as well as neurological disorders and nervous system homeostasis. The mosaics (FIG. 9E) depict the activation state of specific components in each disease/function group. The changes associated with J147 are related to decreases in neurological disease and increases in neuronal function and cancer signaling. The whole transcriptome data also correlated well with the data obtained using the Nanostring technology for inflammatory genes.

In summary, the RNA analysis strongly complements the rest of the data presented here and further supports the potential protective effects of J147 on the central nervous system (CNS) by virtue of its ability to rescue specific aspects of aging that are associated with CNS dysfunction with particular relevance to AD.

Example 8

Discussion

An integrated multiomics approach was used to investigate the potential therapeutic properties of J147 in the SAMP8 model of aging and early sporadic AD. The data show that the detrimental changes in behavior that occur with age are accompanied by alterations in protein and RNA expression, as well as in the levels of many key metabolites. These results identified a large set of parameters in old SAMP8 mice that are associated with stress, vascular pathology, and inflammation that are also observed in human aging and AD. J147 prevented the alteration of many of the metabolic parameters of aging as well as features directly related to the clinical hallmarks of AD, including memory impairment, A β content and tau hyperphosphorylation.

SAMP8 mice develop a progressive, age-associated decline in brain function as well as pathophysiological features similar to those found in the brains of sporadic AD patients. Therefore, they may represent an excellent model for studying the relationship between aging and sporadic AD [6, 7]. The data greatly expand the existing knowledge of the age and AD-associated SAMP8 phenotype at both the brain and system levels. Since the positive effects of J147 in old SAMP8 mice included an improvement in physical and cognitive parameters, reflecting a preservation of their health in general, the results indicate that J147 might be acting by preventing specific metabolic changes that result as a consequence of old age-associated stress.

HSPs represent a major cellular defense against the proteotoxic stress that is characteristic of age-related neurodegenerative disorders. HSP expression depends upon the type of HSP, the disease, cell type and brain region [24]. The changes in HSP40, 60 and 90 observed in the hippocampus of old SAMP8 mice are indicative of stress, and J147 returned the levels of HSP60 and 90 to those of young mice. eIF2 α is also involved in protein homeostasis and potentially AD [10]. J147 returned both its level and phosphorylation state to those of young animals.

One of the most prominent manifestations of stress during aging is the production of inflammatory mediators accompanied by metabolic alterations. Although clinical trials with a few anti-inflammatory drugs failed to prevent AD disease progression, epidemiological studies suggest that long-term use of anti-inflammatory drugs may reduce the risk [13]. The data presented here show an increase in inflammatory parameters in old SAMP8 mice. In the CNS, inflammation is often characterized by the activation of glial cells, mainly astrocytes and microglia. This age-associated phenotype is characteristic of the AD brain [25]. SAMP8 mice also develop astrogliosis [9], and it is shown here that J147 reduces astrocytic reactivity.

Microglia are the resident macrophages of the brain and play a central role during inflammation in the aging and AD brains [26]. Although J147 did not reduce the increased number

of microglia cells found in the hippocampus of old SAMP8 mice, it reduced activation of the stress-induced SAPK/JNK as well as the RNA expression of a number of markers of inflammation in the brain.

Interestingly, the altered expression of several components of the complement system in old SAMP8 mice was not changed by J147. This is important given that the complement system is also activated in human AD patients but that it is thought to be part of a neuroprotective response that helps clear apoptotic cells and A β peptide [27]. Therefore, there are parts of the innate immune system that may be beneficial in the context of aging and AD. The fact that J147 did not alter the levels of these components yet reduced A β levels, inflammation and vascular pathology supports this idea.

A potentially harmful aspect of inflammation in the aging brain is that it may lead to the impairment of vascular integrity and alterations in neuronal homeostasis [14]. Accordingly, aged SAMP8 mice display disrupted BBB permeability [15]. The data indicate that neurovascular dysregulation is an important pathophysiological feature of brains from old SAMP8 mice, and that J147 may protect brain function in these old mice at least in part by preserving BBB homeostasis. The evidence includes the reduction in the levels of VCAM-1, the prevention of IgG infiltration into the hippocampus and the modulation of eicosanoids that regulate vascular dynamics.

J147 significantly increased the levels of DHA in the brain. DHA is the primary structural fatty acid in the human brain and has been linked to cognitive performance. While low plasma levels of DHA are associated with cognitive decline in elderly and AD patients, higher DHA intake and plasma levels inversely correlate with AD risk [28]. DHA supplementation in aged animals enhances learning and memory, and protects against A β and tau pathology in AD mouse models [28-30]. The increase in DHA with J147 could be a consequence of its reduced oxidation, as suggested by lower levels of its oxidized metabolites HDoHes. Furthermore, a dramatic reduction of other oxidized eicosanoids was also observed with J147 treatment.

Aging is accompanied by strong metabolic alterations at the organismal level that are often associated with mitochondrial dysfunction [22, 31]. Analysis of the metabolomic profile of the plasma of old SAMP8 mice revealed profound changes in several biological pathways in comparison to the young SAMP mice. The levels of several amino acids and peptides were found lowered in the old SAMP8 mice. These included γ -glutamyl amino acids, dipeptides and the BCAAs. γ -glutamyl amino acids are generated during the γ -glutamyl cycle, which is involved in the transport of glutathione (GSH) between different organs [32]. GSH is the major intracellular antioxidant. Extracellular GSH is usually broken down to its constituent amino acids by the enzyme γ -glutamyl transpeptidase (GGT) and then those are transported across the plasma

membrane to regenerate GSH intracellularly [32]. This enzymatic reaction transfers the γ -glutamyl moiety from GSH to acceptor amino acids. The fact that several γ -glutamyl amino acids were lower in the plasma of old SAMP8 could be related to impaired GSH homeostasis in the mice.

Amino acid metabolism is of particular relevance to aging because studies with animals and humans strongly suggest that dietary supplementation with essential and branched chain amino acids positively affects physical health and promotes survival [33]. It was proposed that the positive effects of BCAAs are directly associated with improvement of mitochondrial function [33]. J147 restored to young levels many of the alterations in γ -glutamyl amino acids and BCAAs found in the old SAMP8 mice, as well as the levels of several dipeptides.

In a study that compared the plasma metabolome of young with older mice, there was an increase in fatty acids (C18:0 and C20:4 ω 6) with age [34]. Similarly, old SAMP8 mice have significantly increased levels of both C18:0 and C20:4 ω 6 (Table 1) as well as many other fatty acids, in particular a large group of PUFAs. However, in contrast with the earlier report, striking increases in the levels of several acylcarnitines were observed. This is an important observation because fatty acids must be transported into the mitochondria using the carnitine shuttle in order to produce energy via β -oxidation. When mitochondrial β -oxidation is defective, plasma levels of acylcarnitines rise, as observed in old SAMP8 mice. That J147 restored the levels of acylcarnitines suggests a positive effect on mitochondrial dynamics. In addition, it has been shown that acylcarnitines can directly activate pro-inflammatory pathways [35], supporting the idea that J147 also reduces stress-associated inflammation in old age at the systemic level. These data are strengthened by the IPA, which identified diseases related to hepatic dysfunction and inflammatory responses associated with these metabolic alterations.

The analysis of brain metabolites revealed an alteration in glycolysis and the TCA cycle in old SAMP8 mice, further supporting the idea that mitochondrial function is affected with old age. The data are in accordance with a recent study comparing the metabolome of plasma and CSF of AD patients and cognitively normal age-matched controls, where disturbances in multiple pathways related to energy metabolism and mitochondrial function were identified [36].

J147 had an impact on some of the metabolites within the brain, but this was not as pronounced as those found in the plasma. However, it did restore the reduced levels of glutamate detected in old mice to that of young animals. Glutamate can be synthesized from the TCA intermediate α -ketoglutarate, which was also restored by J147. Glutamate is the major neurotransmitter in the brain and is involved in learning and memory processes. Studies have shown a decrease in brain glutamate levels with aging [37], as well as in the AD brain [38, 39].

Another metabolite altered in the hippocampus of old SAMP8 mice whose levels were restored by J147 was cAMP. Upregulation of cAMP signaling has been implicated in AD physiopathology and increased cAMP-PKA signaling in the aging brain is associated with impaired cognition and increased vulnerability to neurodegeneration [23]. PKA is a tau kinase and its dysregulation might be partially responsible for AD-related abnormal tau phosphorylation [40], which could explain in part its increased phosphorylation in the hippocampus of old SAMP8 mice and the protection by J147. PKA signaling was also one of the canonical pathways predicted by the RNA IPA to be affected in old mice.

The relevance of cAMP in the old SAMP8 mice and the therapeutic effect of J147 were validated by the transcriptomic analysis, which identified the cAMP and CREB signaling pathways as being significantly altered. This is an example of how omic approaches that target different cell physiological components can complement each other in order to provide solid readouts about relevant pathways. Changes given by J147 were associated with both the cAMP and CREB signaling pathways, indicating that J147 might be exerting its protective effects in the brain by preserving the proper function of these pathways. The latter result is in agreement with our previous data showing that CREB, a transcription factor with a crucial role in neuronal plasticity and memory, is activated by J147 [3].

Cancer was one of the top diseases associated with aging predicted by the IPA from both the RNA and metabolite data. Although no indication of tumorigenesis was observed in the SAMP8 model, the incidence of cancer increases with age [41]. The changes reported here may thus reflect metabolic alterations associated with cellular senescence, a known contributor to cancer development [41].

Both the metabolomic and transcriptomic analyses revealed that the vast majority of the changes associated with J147 treatment rescued physiological alterations that were observed with aging. These findings strongly indicate that J147 lowers the AD-related pathology in old SAMP8 mice by preventing some of the deterioration associated with aging.

This study combined data derived from different technological approaches to assess the relationship between aging and the therapeutic effects of J147 in the SAMP8 mouse model of aging and sporadic AD. In this regard, the RNA data showed alterations associated with behavior, dysfunction of the nervous system and neurological disease. These predictions given by the IPA not only are in agreement with those found in the metabolite analysis, but also are consistent with the cognitive impairment assessed by the behavioral testing and the changes in proteins required for synaptic function and relevant to AD assessed by Western blotting. The IPA also identified altered pathways in the old SAMP8 mice linked to vascular homeostasis, which are consistent with the

increased vascular inflammation and BBB disruption found with aging, as well as with the changes in eicosanoids involved in vascular function.

Overall, the integration of the data acquired with the multiple scientific techniques applied here not only allowed us to identify with a high level of confidence specific pathologies and molecular pathways characteristic of old age and critical in AD, but also to define a subset that are reverted to youthful levels by J147, indicating that J147 may be effective at treating the primary causes of the disease.

Example 9

AMPK is activated *in vivo*

J147 activates the AMPK/mTOR pathway via its interaction with ATPsyn/MTP, leading to neuroprotection from glutamate and A β toxicity. J147 has therapeutic efficacy in mouse models of familial AD (hFAD), the APP^{swe}/PS1 Δ E9 line 85 mice (Chen et al., PloS one 6, e27865, 2011; Prior et al., Alzheimer's research & therapy 5, 25, 2013) as well as the SAMP8 model of rapid aging and sporadic AD (see examples above). To determine if the same pathways are activated by J147 in these mice, hippocampi of mice fed J147 for 6 mo were assayed by Western blotting for the phosphorylation of AMPK and mTOR. FIG. 10 shows that in all cases AMPK is more phosphorylated, while there are no statistically significant changes in the phosphorylation of mTOR. Thus, J147 can be used to decrease mTOR activity.

Example 10

Materials and Methods

This example describes the materials and methods used to generate the results described in Examples 11-16.

Cell Lines and Tissue

Mouse hippocampal HT22 neural cells are derived from the HT4 cell line and were propagated in DMEM supplemented with 10% FBS. Primary cortical neurons were prepared from embryonic day 17 Sprague-Dawley rats as described (Li et al., J. Cell Biol. 139:1317–24, 1997, Abe et al., Jpn. J. Pharmacol. 53:221–7, 1990). After dissociation from brain tissues with trypsin and DNase I (Sigma), cells were maintained in DMEM with 10% FBS. Induction of intracellular amyloid toxicity in MC65 neural cells (human) was performed as described previously (Liu et al., J. Neurochem. 105:1336–45, 2008).

Cell Viability and Acute Toxicity

Cell viability was determined by MTT assays in 96-well plates. The MTT assay measures the ability of cells to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), as a correlate for cell viability (Li *et al.*, *J. Cell Biol.* 139:1317–24, 1997, Davis & Maher, *Brain Res.* 652:169–173, 1994). Oxytosis, iodoacetic acid (IAA), and A β toxicity assays were performed as previously reported (Liu *et al.*, *J. Neurochem.* 105:1336–45, 2008).

SAMP8 Transcriptome/Metabolome Drift Analysis

Senescence accelerated mouse prone 8 (SAMP8) mice were acquired from Harlan Laboratories (U.K.) and used as previously described for whole transcriptome and metabolomics analysis (Currais *et al.*, *Aging*. 7:1–19, 2015). Young (3 months) and old (10 months) male SAMP8 mice were fed with either control diet (LabDiet 5015, TestDiet, Richmond, IN) or J147 diet (LabDiet 5015 + 200ppm J147, TestDiet). The dose of J147 (~10mg/kg per day) previously proved effective in AD transgenic mice (Currais *et al.*, *Aging Cell* 11:1017–26, 2012). Transcriptional drift analysis was performed as previously described (Rangaraju *et al.*, *Elife* 4:1–39, 2015) with the exception that we removed expressed genes below the 20th percentile. All samples were normalized by setting the over-all mean transcriptional drift to 0 to avoid differences in sample counts that affect drift-variance across samples. Metabolomic drift variance serves as a measure for how closely a metabolome of an old animal resembles the metabolome of a young animal. Metabolomic drift variance is calculated by first determining the metabolomics drift (md) of each individual metabolite (md=log(old/young)). The metabolomics drift variance is then determined by calculating the variance of md across the entire metabolome or subgroups of the metabolome.

DARTS

HT22 cells were plated at 5×10^4 cells/ml and treated with 10 μ m and 50 μ m J147 overnight. The following day, cells were lysed using M-PER (Pierce, 78503) with the addition of protease inhibitors and phosphatase inhibitors (Roche, 11697498001 and 4906845001). Lysate was spun down at 14K RPM for 15 min at 4°C and protein concentration was determined using the BCA Protein Assay Kit (Pierce, 23227). Lysate concentrations were adjusted with M-PER to equivalent protein levels, and digestion was performed using Pronase (Roche, 10165921001) for 10 min at room temperature. Digestions were stopped with 0.5M EDTA (1:19), and samples were immediately put on ice. The resulting digests were separated by SDS-PAGE and visualized independently by silver stain (Shevchenko *et al.*, *Anal. Chem.* 68:850–8, 1996) and Coomassie blue staining. Unique bands in J147-treated samples (representing putative protein targets spared

from proteolysis) as compared to matched control lanes were excised, trypsin-digested, and subjected to liquid chromatography- tandem mass spectrometry (LC-MS/MS). MS results were searched using ScaffoldTM Proteome Software 2.0. Significant identifications were required to have at least two peptides with Sequest Xcorr values of 2.0.

5

J147 Precipitation Pull-Downs

Biotin-J147 was used in pull-down experiments to identify interacting protein targets. HT22 cells and adult male mice subventricular zone (SVZ) brain samples were lysed in lysis buffer (20mM HEPES, 50mM KCl, 20mM MgCl₂, 20mM Na₂MoO₄, 0.1% NP40) on ice. Lysates were
10 precleared with streptavidin magnetic beads (Pierce, 88816), followed by incubation at 4°C overnight with 10µm J147 Biotin-J147, with and without unlabeled “cold” J147 (100µm) to assay competitive target binding. The following day, the bead-J147 complexes were washed with lysis buffer 3x for 10 min., eluted with sample buffer, and run on an SDS-PAGE gel. Whole lanes for each condition were cut into 12 pieces and the proteins from each excised from the gel and
15 submitted to LC/MS/MS for protein identification (Shevchenko *et al.*, *Anal. Chem.* 68:850–8, 1996).

Immunofluorescent Staining

HT22 cells were plated at 2x10⁵ on glass coverslips in 24-well plates and grown overnight
20 (37°C/10% CO₂). The following day, cells were treated with 20 µm Coumarin-J147 (CJ147) or Biotin-J147 (BJ147) for various times during a 24 hr. time-course. After treatment, CJ147- treated samples were washed in warm (37°C) phosphate buffered saline (PBS) for 3x 30 sec. and immediately fixed in warm (37°C) 1% paraformaldehyde (PFA) for 10 min. at 37°C. Cells were then washed 3x for 10min in PBS, and two drops NucRed® Live 647 ReadyProbes® Reagent
25 (ThermoFisher, R37106) were added prior to mounting in Fluoro-Gel (EMS, 17985-10). BJ147- treated samples were treated similarly prior to fixation, then 2% PFA was used for fixation, followed by permeabilization with 0.5% Triton-X in PBS for 10 min. and overnight incubation in blocking buffer (10% donkey serum in 0.1% Triton-X in PBS) at 4°C. The following day, anti-COXIV (1:500, Cell Signaling, 4844) diluted in blocking buffer was added for 2 hrs. at room
30 temperature (RT), followed by incubations with donkey anti-rabbit 568 and streptavidin-488 (ThermoFisher, A10042 & S11223) for 1 hr. at RT. Cells were washed 3x in PBS before the addition of DAPI (1µg/ml, Sigma, D9542) and mounting in Fluoro-Gel. Images were acquired using a Zeiss LSM 710 confocal immunofluorescence microscope.

Complex V Activity Assay

Complex V activity was assayed using the MitoTox OXPHOS Complex V Activity Kit (Abcam, ab109907) per manufacturer's instructions. An inactive derivative of J147, CAD120 (100nm) and DMSO were used as negative controls, and oligomycin (10µm) (Sigma, 75351) as a positive control. The reaction was carried out in DMSO (solvent for J147), J147, or CAD120 and variable amounts of ADP. ATP synthesis was monitored by the increase/decrease in luminescence over time by a Spectramax M5 plate reader using luminescence detection mode. The initial velocity of ATP synthesis was calculated from the slope of the initial reaction. Enzyme inhibition kinetics were analyzed by nonlinear regression least-squares fit using GraphPad Prism.

Western Blot

Western blots were performed as previously described (Currais *et al.*, *Aging Cell* 13:379–90, 2014). Antibodies used were as follows: APP C-terminal (Sigma, A8717), ATP5A (Abcam, 14748), pAMPK (Cell Signaling, 2535), AMPK (Cell Signaling, 2793), pS6 (Cell Signaling, 4858), S6 (Cell Signaling, 2317), pRaptor (Cell Signaling, 2083), Raptor (Cell Signaling, 2280), pACC1 (Cell Signaling, 11818), ACC1 (Cell Signaling, 4190), Actin (BD Transduction LaboratoriesTM, 4125). Horseradish peroxidase-conjugated secondary antibodies: goat anti-rabbit, goat anti-mouse (1:5,000, Bio-Rad, 1706516, 1721019). The CamKK2 inhibitor STO-609 (Cayman 15325) was used at 1µg/ml.

ROS and Membrane Potential Measurements

Superoxide (Molecular Probes, MitoSox M36008) and mitochondrial membrane polarity (Molecular Probes, JC-1 T3168) experiments were performed according to the manufacturer's instructions. Briefly, HT22 cells were plated at 5x10⁵ in black-walled 96-well plates (Corning) and grown in phenol red-free media (ThermoFisher FluoroBrite DMEM A1896702) supplemented with glutamine (4mM) at 37°C/10% CO₂ for 24 hrs. until confluent. J147 was added to cells for at least 1 hr. before addition of dyes. JC1 (1µg/ml) and MitoSox (2.5µm) were added to cells for 45 min. and 15 min, respectively at 37°C. Fluorescent measurements were immediately read on a Spectramax M5 plate reader (Molecular Devices). Excitation/emission settings were as follows: JC-1: monomer – 488±10nm/530±10nm; aggregate – 535±20nm/590±20nm. MitoSox: 510/580nm. JC-1 monomer and aggregate fluorescence were measured independently. Oligomycin (1µg/ml) and FCCP (10µm) (Sigma, C2920) were used as positive and negative controls.

Cytosolic and Mitochondrial Calcium Measurements

Rhod-2 and Fluo-4,AM (Molecular Probes, R1244 and F14201) were used according to the manufacturer's instructions to measure mitochondrial and cytosolic Ca²⁺ levels, respectively. HT22 cells were plated at 5x10³ cells/well in black-walled 96-well plates in FluoroBrite DMEM and grown overnight at 37°C/10% CO₂. The following day, glutamate (2mM, 5mM, 10mM) (Sigma, G1251) and/or J147 were added for 6-8 hours prior to addition of calcium dyes. For calcium ionophore experiments, A23187 (Tocris 1234) and ionomycin (Cayman 10004974) were added at the indicated concentrations along with J147 for 1 hr. Pluronic F-127 (Molecular Probes, P6866) was used to assist in Fluo-4 and Rhod-2 dispersion and used at a final concentration of 0.02%. Fluo-4 (2.5µm) loading solution was added to cells for a total of 45 min., the first 20 minutes at RT, and the last 25 min. at 37°C, as has been previously suggested (Bootman *et al.*, *Cold Spring Harb. Protoc.* 1:122–5, 2013). Rhod-2 (2.5µm) was chemically reduced with excess sodium borohydride prior to cell loading. Cells were incubated with Rhod-2 for 30 min at 37°C, then rinsed 2x with PBS, replaced with fresh media, and returned to the incubator for an additional 30 min. to allow for deesterification of intracellular AM esters. Fluorescence was either measured on a Spectramax M5 plate reader, excitation/emission: Rhod-2: 552/582nm and Fluo-4: 488/516nm or by flow cytometry, in which DAPI (1µg/ml) was added prior to analysis using FITC and PE detectors gated on live cells using a BD LSRII. FloJo software was used for analysis, and results reported as geometric mean fluorescence intensity.

ATP and NAD/P/H

ATP measurements were carried out according to the manufacturer's instructions (Molecular Probes A22066, Promega G9071 and G9081). For ATP measurements, HT22 cells were plated at 3x10⁵ cells/60mm dish and grown overnight at 37°C. The following day, cells were treated with the indicated concentrations of J147 for 4 hrs and processed as described. Results were normalized to the protein concentration, and are reported as either nmol ATP/µg protein or relative fold-change. Cells for NAD/H and NADP/H measurements were plated at the same density, but were scraped into ice-cold PBS and lysed with 1:1 of base solution with 1% DTAB. Results are reported as fold-changes relative to control conditions. For toxicity experiments, cells were treated with IAA and J147 for 2 hrs., after which media was replaced with only J147-containing media for an additional 2 hours prior to harvest. Luminescence was measured on a Spectramax M5 plate reader, using a 9-point well scan method.

Luciferase Reporter Assay and Transfections

For transfections and siRNA knockdowns, HT22 cells were plated at 5×10^5 in 60 mm dishes and grown for 24 hrs. prior to transfection. For transfections, 2.5 μ g of a pGL3 firefly luciferase control vector or reporter constructs encoding either a NF- κ B response element or β -galactosidase (β -gal) reporter were transfected using Lipofectamine 3000 Reagent (Invitrogen L3000001) for 6-8 hrs., after which media was replaced and cells were grown overnight. siRNA transfections using 50 nm ATP5A siRNA and 50 nm control siRNA (Santa Cruz sc-60228 and sc-37007) were performed similarly using RNAiMAX Reagent (Invitrogen 13778) and grown overnight. For experiments measuring NF- κ B activity in ATP5A knockdown cells, co-transfections were performed for 6-8 hours followed by media replacement. The following day, transfected cells were trypsinized and seeded at 3×10^5 for use in experiments. Cells were treated with the indicated concentrations of J147 for 4 hrs. before lysing in 1X Reporter Lysis Buffer (Promega E3971). Luciferase assays were performed using the Luciferase and Beta-Glo systems (Promega E1483 and E4720) following manufacturer's instructions. 9-point well-scan readings were performed in white-walled plates (Costar 3912) using a Spectramax M5 plate reader. Results were normalized to β -gal and reported as fold-changes relative to control/untreated conditions.

Drosophila Stocks, Culturing Conditions, and Lifespan Analysis

The Canton-S and w1118 lines have been described previously and F1 offspring from crosses between the two strains (w1118/+) were used (Ratcliff *et al.*, *PLoS One* 10:1–20, 2015). Male flies were collected and aged in same-sex cohorts (25 flies per vial) on standard lab media (agar, molasses, yeast, cornmeal, propionic acid, nipagin). Starting at 1 week of age, flies were placed on to standard fly media (control) or vials containing standard media containing 0.1 μ M or 2 μ M J147 (final concentration). Flies were maintained at 25°C in 50-60% humidity on a 12hr:12hr light:dark cycle and turned on to fresh media and the number of dead flies recorded, 3 times weekly for the duration of the study. Mortality data was used to generate Kaplan-Meier longevity curves.

Example 11

Identification of molecular target of J147

To identify the molecular target of J147, the unbiased small molecule target identification approach of drug affinity responsive target stability (DARTS) was used to detect putative binding partners (Lomenick *et al.*, *Curr. Protoc. Chem. Biol.* 42:157–62, 2011). Lysates from the HT22 hippocampal neural cell line were incubated with vehicle, 10 μ M or 50 μ M J147 for 15 minutes before treating with pronase to degrade unbound protein complexes. Lysates were then resolved on

SDS-PAGE and stained with Coomassie blue to identify bands containing non-degraded proteins specific to J147 treatment. Mass spectrometry (MS) analysis on excised bands identified α -F1-ATP synthase (ATP5A) and Hsp60 (HSP60) as putative targets (FIG. 11A, arrow). Interestingly, both proteins are found in the mitochondria, where ATP synthase controls the synthesis/hydrolysis of ATP, and Hsp60 regulates the import of folded proteins into the mitochondria (Bross *et al.*, *Curr. Top. Med. Chem.* 12, 2491–503 (2012); Cossio *et al.*, *Uma ética para quantos?* XXXIII, 81–87, 2012). These results indicated that J147 targets a subset of mitochondrial proteins.

To provide further evidence for ATP5A or Hsp60 as molecular targets of J147, HT22 cells and subventricular zone (SVZ) tissue lysates were incubated with a biotinylated derivative of J147, BJ147. The BJ147 derivative is neuroprotective like J147. Following overnight incubation with streptavidin conjugated magnetic beads, eluted lysates were run on SDS-PAGE gels and LC (liquid chromatography)/MS/MS protein identification of entire lanes was performed. Consistent with the DARTs experiment, a strong enrichment of mitochondria-associated proteins was present in streptavidin pull-down fraction from BJ147- incubated samples. These included proteins involved in ion flux and transport, such as the F1- α and - β -ATP synthases, inositol 1,4,5- triphosphate receptor 3 (InsP3R3), members of the solute carrier family 25 (SLC25a3-5), and voltage-dependent anion channel (VDAC) (FIG. 11B, 12A, and 12B). These proteins have all been suggested to comprise and/or regulate the mitochondria permeability transition (mPT) pore (Baines, *J. Mol. Cell Cardiol.* 46:850–7, 2009; Deniaud, *et al.*, *Oncogene* 27:285–299, 2008), a complex of mitochondrial membrane-associated proteins responsible for the initiation of cell death programs during conditions of toxic stress (Rego *et al.*, *Cell Death Differ.* 8:995–1003, 2001). ATP synthase is reported to be an indispensable core component of the mPT pore (Bernardi *et al.*, *Physiol. Rev.* 95:1111–1155, 2015; Giorgio *et al.*, *Proc. Natl. Acad. Sci.* 110:5887–92, 2013) and the amount of ATP synthase was greatly reduced in BJ147-precipitated lysates incubated with 10X unlabeled J147 as a binding competitor (FIG. 11C).

Example 12

J147 targets mitochondrial metabolism by modulating ATP synthase activity

The intracellular localization of J147 in HT22 cells was determined by confocal fluorescent microscopy using BJ147 as well as a fluorescent coumarin derivative of J147 (CJ147). An imaging time course demonstrated that J147 localized to the mitochondria (FIG. 13A), and co-localized with cytochrome c oxidase IV (COXIV) (FIG. 13B). Localization to the mitochondria was rapid, occurring within 10 min. of J147 addition to cell cultures. Thus, both biochemical and localization experiments support the mitochondria as a target of J147.

One of the most highly enriched mitochondrial proteins in both the DARTS and affinity precipitation experiments was ATP5A, indicating it was a putative target. Therefore, it was determined whether ATP synthase activity is modulated by J147. J147's effect on ATP synthase enzyme kinetics was determined in isolated bovine heart mitochondria. In this assay, ATP hydrolase activity is coupled to the conversion of NADH to NAD⁺, allowing detection of decreasing absorbance at OD 340 nm, indicative of a decrease in ATP hydrolysis. A dose response curve is shown after 1hr of J147 incubation (FIG. 13C), indicating saturating partial inhibition ($23.6 \pm 3.4\%$) of ATP hydrolysis with an EC₅₀ of 20nM. Oligomycin, an inhibitor of both forward (ATP synthesis) and reverse (ATP hydrolysis) ATP synthase activity, and an inactive derivative of J147 served as positive and negative controls, respectively. These results demonstrate that J147 binds to and modulates the activity of mitochondrial ATP synthase.

The F1- α -ATP synthase subunit comprises a part of the inner mitochondrial membrane portion of the ATP synthase complex, also referred to as Complex V of the electron transport chain (ETC). The ATP synthase complex couples the production of ATP or its hydrolysis to ADP and AMP to the transport of H⁺ ions across the inner mitochondrial membrane, ensuring it remains hyperpolarized to preserve cell viability. However, conditions of stress can result in membrane depolarization and loss of membrane potential ($\Delta\psi_m$), leading to mPT pore opening and subsequent cell death. J147's effect on $\Delta\psi_m$ was tested using JC1, a cationic ratiometric dye that shifts fluorescence emission from green (~529 nm) to red (~590 nm) upon aggregation in hyperpolarized mitochondria. A small but significant dose-dependent increase in mitochondrial membrane potential is observed within 1 hr of J147 treatment (FIG. 13D), an effect consistent with regulation of ATP synthase activity. Accordingly, modulation of ATP synthase complex activity via siRNA-targeted knockdown of the α -subunit of ATP synthase elicited a similar increase in $\Delta\psi_m$ (FIG. 13E).

Concomitant with changes in ATP synthase activity is the production of reactive oxygen species (ROS). Traditionally thought of as being detrimental, new evidence indicates that mitochondrial ROS can elicit a protective preconditioning response involving the activation of nuclear factor kappa light polypeptide gene enhancer in B-cells (NF- κ B). In examining this possibility, it was observed that both J147 treatment and ATP5A knockdown caused a slight but significant increase in superoxide levels within the mitochondria as measured by a dose-dependent increase in the fluorescence shift at ~580 nm using the MitoSOX ROS indicator dye (FIG. 13F). Furthermore, when luciferase assays were used to measure NF- κ B reporter activity in HT22 cells treated with J147, an increase in NF- κ B activity was seen at 100 nM and 1 μ M (FIG. 13G). ATP5A knockdown mirrored the J147-induced increase in NF- κ B activity ($n=4$; $p < .05$).

In the elderly and in patients with AD, reduced levels of ATP and NAD⁺ have been linked to mitochondrial dysfunction (Zhang *et al.*, *J. Alzheimers. Dis.* 44:375–378, 2015; Reddy *et al.*, *Trends Mol. Med.* 14:45–53, 2008; Gomes *et al.*, *Cell* 155:1624–1638, 2013). ATP and NAD⁺ are not only key drivers of metabolism, but also critical signaling molecules regulating transcription (Barger *et al.*, *PLoS One* 10, e0120738, 2015) and receptor-mediated signaling (Song *et al.*, *Am J Physiol Cell Physiol.* 310(2):C99–114, 2015). Preserving their levels is thought to promote neuron survival, as supplementation of NAD⁺ prevents neuronal death in a toxic misfolded prion protein (TPrP) mouse model (Zhou, M. *et al.* Neuronal death induced by misfolded prion protein is due to NAD⁺ depletion and can be relieved in vitro and in vivo by NAD⁺ replenishment. *Brain* 138, 992–1008 (2015). Dose response curves indicate that J147 increased both ATP and NAD⁺ levels in HT22 cells within 4–6 hrs of treatment (FIGS. 13H, 13I). Furthermore, ATP5A siRNA-targeted knockdown similarly increased whole cell ATP levels in these cells (n=3; p < .05).

Example 13

Modulation of ATP synthase protects from neurotoxic insults

It was next determined whether modulating ATP synthase activity would elicit a similar neuroprotective effect as J147 in assays modeling old age-associated toxicities that contribute to dementia, such as AD. If so, modulating ATP synthase activity by siRNA-targeted knockdown should protect in models of A β toxicity (proteotoxicity), oxytosis (glutamate toxicity), and ischemia (IAA toxicity). In fact, modulation of ATP synthase activity induces pro-survival responses and extends lifespan in a variety of organisms.

To determine a functional role for ATP5A in the acute toxicity assays, whether siRNA targeted to the α -subunit of ATP synthase protects from A β proteotoxicity was determined. Human MC65 nerve cells conditionally express the C99 fragment of amyloid precursor protein (APP) under the control of a tetracycline (tet) promoter. Upon induction by removal of tetracycline, C99 is processed to produce A β polymers leading to cell death, an effect that is blocked by J1475. Similarly, ATP5A knockdown also prevented intracellular A β -induced cell death in MC65 cells (FIG. 14A).

The neuroprotective role of ATP5A was determined in toxicity models of oxytosis and ischemia. High levels of extracellular glutamate block cystine import resulting in glutathione depletion, aberrant Ca²⁺ flux and cell death during oxytosis, while IAA irreversibly inhibits the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to induce ischemia. J147 protects HT22 cells from oxytosis and IAA toxicity (Chen *et al.*, *PLoS One* 6, e27865, 2011), and it

was demonstrated here that knockdown of ATP5A (FIG. 14B) mediates a similar protection in both assays (FIGS. 14C, 14D). However, cell viability is not further improved in ATP5A siRNA-targeted knockdown cells by J147 in the oxytosis toxicity model, indicating that ATP synthase is required for the neuroprotective effect of J147 (FIG. 14E). Furthermore, J147 partially restored the levels of both NAD⁺ and ATP in the IAA-induced ischemia toxicity model (FIGS. 13I, 14F), indicating that preservation of ATP and NAD⁺ may be part of the neuroprotective response elicited by J147.

An influx of Ca²⁺ is involved in many forms of neural cell death, including oxytosis in HT22 cells. Toxic increases in Ca²⁺ levels have been reported to induce Ca²⁺ overload within the mitochondria, causing opening of the mPT pore and cell death. Because of recent reports suggesting ATP synthase is a core component of the mPT pore, it was determined whether J147 itself or ATP5A knockdown mitigates toxic Ca²⁺ flux in the oxytosis toxicity model. Indeed, after 10-12 hrs of glutamate exposure, a substantial rise in Ca²⁺ is observed in both the cytosol and the mitochondria as indicated by fluorescent increases in Fluo-4 and Rhod-2 Ca²⁺-indicator dyes (FIG. 14G). J147 prevented this toxic Ca²⁺ influx in both the cytosol and mitochondria (FIG. 14G). Similarly, ATP5A knockdown prevented this glutamate-induced Ca²⁺ increase in the mitochondrial compartment (FIG. 14H). J147's role in mitigating Ca²⁺ influx was determined via addition of the Ca²⁺ ionophores ionomycin and A23187. Both increased cytosolic and mitochondria calcium levels, which were dampened in the presence of J147 (FIGS. 12A and 12B). These results indicate a central role for the F₁F₀-ATP synthase in mitochondrial Ca²⁺ flux and demonstrate that this complex mediates neuroprotection in the acute toxicity assays.

Together these data demonstrate that reduced levels of ATP5A are neuroprotective in aging-associated toxicity assays, including A β and IAA-induced toxicity as well as oxytosis, phenocopying the protective effects of J147.

Example 14

J147 modulates AMPK/mTOR signaling

Since age is the greatest risk factor for AD, interventions that slow aging or extend healthspan might serve as therapies that delay disease onset. Recent studies have highlighted a role for ATP synthase in the regulation of mTOR and life span extension in flies and worms. Inhibition of mTOR via activation of AMPK is a canonical longevity-associated pathway, as it has been shown to prolong lifespan in a number of diverse animal models (Mair & Dillin, *Annu. Rev. Biochem.* 77:727-754, 2008). The AMPK/mTOR axis serves as a metabolic sensor promoting autophagy, translational inhibition, and survival processes under conditions of energy deprivation.

Activation of AMPK is achieved through phosphorylation of threonine (Thr) 172 on the α -subunit, lowering activity of some ATP- consuming pathways while promoting ATP synthesis through others, such as glycolysis and fatty acid oxidation. Therefore it was determined whether J147 modulated AMPK/mTOR signaling via ATP synthase.

AMPK/mTOR activity was monitored using site-specific phosphorylation antibodies against proteins involved in this pathway. In FIGS. 15A-15D, three different cell types were used to assay the activity of the AMPK/mTOR pathway following treatment with J147. In mouse HT22 cells, human MC65 cells, and primary rat cortical neurons, there is a clear, time-dependent activation of AMPK by 50 nM J147. AMPK phosphorylation of raptor at Ser792 is critical for AMPK- mediated inhibition of mTOR. An increase in raptor Ser792 phosphorylation was observed in all three cell types treated with J147 (FIGS. 15A-15D). Raptor-mediated inhibition of mTORC1 activity reduces unnecessary ATP expenditure by decreasing S6 kinase activity resulting in a halt of cap- dependent protein translation. AMPK-mediated phosphorylation of acetyl-CoA carboxylase (ACC1) increases β -oxidation of fatty acids, promoting energy production. J147 decreases S6 and increases ACC1 phosphorylation (FIGS. 15A-15D) in all three cell types, indicating that the AMPK/mTOR signaling pathway may be a conserved J147-targeted pathway. siRNA-mediated knockdown of ATP5A in MC65 cells phenocopied the effects of J147 on the AMPK/mTOR signaling axis, resulting in increased raptor Ser792 phosphorylation as well as decreases and increases in S6 and ACC1 phosphorylation, respectively (FIGS. 16A-16F).

J147 caused an increase in AMPK phosphorylation despite modestly increasing ATP levels, indicating an alternative mode of AMPK activation to that of sensing the AMP:ADP:ATP ratios. In the brain, calcium/calmodulin-dependent protein kinase kinase β (CamKK2) has been shown to activate AMPK51, pointing to a potential Ca^{2+} -mediated J147 activation of AMPK. Therefore, it was determined if J147 might regulate CamKK2 activity by modulating resting Ca^{2+} homeostasis. Measuring cytosolic and mitochondrial Ca^{2+} levels upon J147 treatment in unstressed cells revealed dose-dependent changes, where increases in cytosolic Ca^{2+} and a decrease only at the highest concentration of J147 in mitochondrial Ca^{2+} levels were observed (FIGS. 17A-17B). To determine if CamKK2 mediates J147 activation of AMPK, primary cortical neurons were treated with J147 and a potent inhibitor of CamKK2, 7-Oxo-7H-benzimidazo[2,1- a]benz[de]isoquinoline-3-carboxylic acid acetate (STO-609). STO-609 prevented J147 mediated activation of AMPK in cortical neurons (FIGS. 15C, 15D), thereby identifying CamKK2 as key mediator of J147 modulation of AMPK/mTOR modulation in cortical neurons. These data implicate the regulation of Ca^{2+} flux as an additional mode of ATP synthase signaling under normal conditions.

Together, these findings implicate Ca²⁺ signaling via CamKK2 activation as an important mediator of J147-induced AMPK/mTOR activity. This indicates that the J147-mediated modulation of ATP synthase has several roles, tempering toxic Ca²⁺ flux in situations of acute stress while under basal conditions maintaining a healthy metabolic profile through modulation of the AMPK/mTOR pathway and increasing ATP and NAD⁺ levels.

Example 15

J147 attenuates age-associated decline and extends lifespan in vivo

The results herein demonstrate that J147 protects from age-associated brain toxicities in cell culture models via interaction with ATP synthase and subsequent modulation of the AMPK/mTOR pathway. It was previously demonstrated that J147 improves age-associated cognitive decline in mouse models of familial AD (APP^{swe}/PS1 Δ E9) and sporadic old-age associated dementia using SAMP8 mice. These previous results, in combination with the findings herein, indicate that J147 may improve age-associated cognitive decline by acting on a canonical aging pathway.

It was determined whether the AMPK/mTOR signaling pathway is activated by J147 in SAMP8 mice and whether J147 induced physiological changes consistent with delayed aging. Hippocampi from mice fed J147 (200ppm; ~10mg/kg/day) for 6 months, starting at 3 months of age, were isolated and used for Western blot analysis of the same AMPK/mTOR targets that were assayed in vitro (FIG. 15A-15D). J147 significantly increased the phosphorylation of AMPK and its substrate ACC1 while differences in the phosphorylation of raptor and S6 did not reach significance (data not shown) (FIGS. 18A, 18B). As phosphorylation of ACC1 promotes energy production via fatty acid β -oxidation, this result supports our previous characterization of the J147-effect on the metabolic profile of aged SAMP8 mice in which we used multimodal bioinformatics to examine global levels of RNA and metabolites in the blood, brain, and plasma⁸. In these mice, levels of multiple acylcarnitines in plasma were increased over young controls, a consequence of defective mitochondrial β -oxidation. J147 reverted this increase, supporting the *in vitro* data demonstrating a J147-mediated induction of AMPK phosphorylation and consequent ACC1 inhibition, likely leading to increased fatty acid entry into the mitochondria and correcting for dysfunctional β -oxidation of fatty acids.

As J147 appears to target a canonical aging pathway, it was determined whether J147 affected aging itself. Recently, it has been shown that aging progressively destabilizes the transcriptome, resulting in a drift in mRNA transcript levels away from those observed in young animals (Rangaraju *et al.*, *Elife* 4:1–39, 2015). As some longevity mechanisms dramatically suppress age-associated transcriptional drift and preserve a youthful transcriptome phenotype

(Rangaraju *et al.*, *Elife* 4:1–39, 2015, Barger *et al.*, *PLoS One* 10, e0120738, 2015), it was determined whether J147 had a similar effect. Analysis of hippocampal gene expression data from 3 and 10 month old SAMP8 mice confirmed that transcriptional drift variance did increase with age across the entire transcriptome (FIG. 18C). However, treatment with J147 attenuated age-associated transcriptional drift, reducing its variance by ~6% ($p < 10^{-10}$) and preserving a more youthful transcriptional profile (FIG. 18C).

Because J147 targets the mitochondria ATP synthase, it was determined whether J147 specifically suppressed age-associated drift of gene transcripts involved in mitochondrial functions. Indeed, J147 dramatically suppressed transcriptional drift of transcripts encoding mitochondrial components of both carnitine metabolism and ATP synthesis coupled proton transport (FIG. 18E). Carnitine is critical for maintaining mitochondrial function and ATP synthesis since it is required for the transport of long chain fatty acids into the mitochondria, resulting in their oxidation and production of acetyl-CoA for entry into the tricarboxylic acid cycle (TCA cycle). Long chain acylcarnitines decrease with age and their over-abundance in the plasma is indicative of mitochondrial dysfunction. It was previously shown that J147 reduces acylcarnitine accumulation in old SAMP8 mice (Currais *et al.*, *Aging* 7:1–19, 2015). This indicates that modulating ATP synthase activity may restrain the age-associated loss of orchestrated gene expression involved in coordinating mitochondrial bioenergetics. These data also indicate a potential feedback loop coupling ATP synthase activity and expression, and show that J147 stabilizes mitochondrial transcripts against the effect of aging.

Similar to assessing transcriptional drift, metabolic drift analyses can be used to monitor aging of the metabolome. Because mitochondria are essential for maintaining metabolic homeostasis, it was determined if J147 had a protective effect on metabolic drift similar to its effect on the transcriptome. Targeted metabolomics was performed on brain and plasma extracts from 3 and 10 month old SAMP8 control or J147 treated mice. At 10 months of age, no significant differences in the brain metabolome were observed between old and young animals. However, a substantial metabolomic drift in plasma metabolites was detected, an effect that was attenuated by J147 treatment (FIG. 18D). This is in agreement with the J147-mediated reversion of increased plasma acylcarnitine levels (Currais *et al.*, *Aging* 7:1–19, 2015), and demonstrates that J147 treatment stabilized the hippocampal transcriptome and plasma metabolome against the age-associated drift that occurs in SAMP8 mice.

It was determined whether J147 could extend longevity in *Drosophila*, where ATP synthase interaction with mTOR has previously been reported to affect lifespan. J147 clearly increased the longevity of male *Drosophila*, with 100 nM and 2 μ M J147 extending lifespan by 9.5% and 12.8%,

respectively (FIG. 18F). Taken together, the *in vivo* data show that the action of J147 is not restricted to the nervous system but extends lifespan by promoting a healthy metabolic profile that attenuates aging across multiple tissues. J147 was identified in phenotypic screens designed to select compounds that prevent age-associated toxicities, and the results suggest it does so by acting on a canonical aging pathway. This is the first demonstration of an interdependent relationship between aging and an AD drug candidate at the molecular level, indicating that aging and AD share common drug targets.

Example 16

Discussion

It is shown herein that the α -F1-subunit of ATP synthase is a high affinity target for J147, mediating protection against acute toxicity in addition to promoting long-term mitochondrial stability. Modulation of ATP synthase activity resulted in changes in Ca²⁺ homeostasis and activation of CamKK2, leading to AMPK activation and inhibition of mTOR. The activation of this signaling cascade most likely stabilizes the transcriptome and the metabolome against effects of aging, reduces cognitive decline, and increases lifespan.

J147 was identified by phenotypic screening for potential AD drug candidates in assays modeling age-associated neurotoxicities. It was previously shown that J147 enhanced memory and rescued cognitive decline in mouse models of sporadic dementia (SAMP8) and familial AD APP^{swe}/PS1 Δ E9. It was superior to Aricept in spatial memory tests, and reversed AD pathology in old symptomatic mice (Currais *et al.*, *Aging* 7:1–19, 2015). Thus, screening for neuroprotective effects *in vitro* led to a compound that modulates a canonical aging pathway. J147 acts on the same target as α -ketoglutarate, a metabolite identified through phenotypic screening to extend life span in *C. elegans* (Chin *et al.*, *Nature* 510:397–401, 2014). Previous studies have revealed that interventions that extend lifespan also show beneficial effects in models of neurodegenerative disease (Ng *et al.*, *Front. Cell. Neurosci.* 9:1–13 (2015); Gan *et al.*, *Aging Cell* 1:924–9 (2010); Partridge *et al.*, *Oncogene* 27:2351–63 (2008)). However, the results herein indicate that the reverse can also be true, that interventions that improve cognitive decline may also extend lifespan. Interestingly, screens for compounds that extend lifespan have repeatedly resulted in drugs like lithium, valproate, antipsychotics or antidepressants that are currently being used to treat neurological disorders (Rangaraju *et al.* *Aging Cell* 1:971–981 (2015)). The identification of a drug target suitable for aging as well as age-associated cognitive decline indicates that the two are more closely related on a molecular level than previously thought (Riera & Dillin *Nat. Comments* 21:1400–5 (2015); Bredesen, *Aging* 6:707–17 (2014)).

The identification of ATP synthase as a suitable drug target for dementia emphasizes the utility of employing phenotypic screening to identify potential compounds and their targets that exert systemic age-protective effects. Conventional target validation approaches would have excluded ATP synthase as a target because genetic mutations in several subunit-coding genes have been implicated in a number of neurodegenerative diseases, such as Leigh's syndrome, bilateral striatal necrosis, Batten's disease, Down syndrome, and even AD64–70. However, saturating amounts of J147 still only result in 20% reduction in ATP hydrolase activity without causing deleterious effects.

Mitochondria regulate a variety of metabolic signaling pathways and are involved in programs of cell survival and death. They are uniquely poised to integrate calcium signals with energy metabolism in order to coordinate many cellular processes, including but not limited to, protein translation, autophagy, retrograde nuclear signaling, and ATP and antioxidant production⁴. Within the past two decades, evidence has indicated a causal relationship between mitochondrial dysfunction and age-associated phenotypes observed in many neurodegenerative diseases, such as AD (Sullivan & Brown Prog. Neuro-Psychopharmacology Biol. Psychiatry 29:407–410 (2005); Hauptmann *et al.*, Exp. Gerontol. 41, 668–73 (2006); Reddy *et al.*, Biochim. Biophys. Acta 1822, 639–49 (2012); Reddy & Beal, Brain Res. Brain Res. Rev. 49, 618–32 (2005); Naoi *et al.*, J. Neural Transm. 116, 1371–1381 (2009); Bubber *et al.*, Ann. Neurol. 57, 695–703 (2005); Witte *et al.*, Mitochondrion 10, 411–418 (2010); Picone *et al.*, Oxid. Med. Cell. Longev. 2014, 1–11 (2014)). Several observations have documented metabolic deficiencies in the AD brain preceding neuropsychological impairment and atrophy, where levels of key mitochondrial enzymes involved in oxidative phosphorylation (OxPhos) are altered. Furthermore, studies have demonstrated A β localizes to the mitochondria and binds specific mitochondrial-associated proteins, resulting in aberrant mitochondrial bioenergetics (Dragicevic *et al.*, J. Alzheimers. Dis. 20 Suppl 2, S535–50 (2010); Caspersen, FASEB J. 19(14):2040-1, (2005); Cha *et al.*, PLoS One 7, e34929 (2012)). This has led to the idea that proper metabolic control is critical for mounting a successful response to the toxic stresses afflicting the aging brain, and may provide alternatives to the amyloid pathway for AD-targeted therapeutic interventions (Picone *et al.*, Oxid. Med. Cell. Longev. 2014, 1–11 (2014)).

While the kinetic data herein demonstrating ~20% inhibition of ATP hydrolase activity indicates a covalent/allosteric regulation of ATP synthase activity, it does not exclude involvement of other protein binding partners. The mitochondrial-associated proteins VDAC, Slc25a, and IP3R3 were also identified in J147-pull-down samples from HT22 cells and SVZ tissue. These proteins, along with α -F1-ATP synthase, have been implicated in the composition of the mPT pore responsible for executing cell death programs during conditions of toxic stress. Being sensitive to

changes in redox potential, Ca^{2+} , voltage, adenine nucleotides, and pH, the mPT pore is a crucial regulator of cell death. In lethal conditions of stress, the mitochondrial buffering capacity to toxic levels of Ca^{2+} influx is lost, leading to mPT pore opening and the release of pro-apoptotic factors that initiate cell death programs. However, among the proteins identified in the pull down experiments, only knockdown of ATP5A prevented cell death in the oxytosis, IAA, and $\text{A}\beta$ -induced toxicity models (FIGS. 14A-14D), indicating ATP5A as a primary target mediating J147's effects against acute toxicity.

More recently, Formentini *et al.* (Embo J 33:762–778, 2014) demonstrated a specific *in vivo* role for ATP synthase in the protection and survival of brain neurons against excitotoxic damage through the generation of a conditional mouse model expressing the human form of mutant ATPase inhibitory factor 1 (hIF1), leading to sustained inhibition of ATP synthase. Endogenous overexpression of IF1 in neurons has been suggested to be a mechanism of survival and protection via rerouting of metabolic pathways. The results herein are consistent with those of Formentini *et al.* demonstrating hIF1-modulation of ATP synthase activity leading to a ROS-mediated retrograde NF- κ B pro-survival response in neurons against quinolinic acid-induced excitotoxicity, as well as the maintenance of $\Delta\psi_m$ after ADP addition. It is shown herein that J147 and ATP synthase knockdown elicited a significant increase in mitochondrial superoxide production and $\Delta\psi_m$ (FIGS. 13E, 13F), potentially explaining the induction of NF- κ B activity observed in both J147 and ATP synthase knockdown conditions (FIG. 13G). Despite a number of conflicting reports describing NF- κ B involvement in both pro-survival and pro-apoptotic initiating events (Karin & Lin, Nat. Immunol. 3, 221–227 (2002)), studies in p50^{-/-} knockout mice suggest that NF- κ B may delay the onset of aging and extend longevity in mice (Lu et al., Neuroscience 139, 965–978 (2006), indicating yet another way in which modulation of ATP synthase activity may contribute to lifespan extension. It is possible that these effects are a consequence of reverse electron flow through the ETC96 caused by unidirectional activity of ATP synthase. Further exploration of this phenomenon is needed to understand the specific role and precise location of ROS generation during J147 treatment.

The data herein indicate that modulation of ATP synthase activity, either by J147 or siRNA-mediated knockdown, initiates a dual-purpose Ca^{2+} -mediated protective cascade that results in both the dampening of toxic Ca^{2+} influx into the mitochondria during acute toxicity (FIGS. 12A, 12B, 14G, 14H) as well as the activation of CamKK2. The observation that J147 slightly increased cytosolic Ca^{2+} levels even under control conditions (FIG. 17A) most likely explains for the activation of CamKK2, leading to an investigation of the role of CamKK2 in AMPK phosphorylation. It has been shown that Ca^{2+} /CaM-mediated activation of CamKK2 leads to

AMPK activation in the brain (Hurley, J. Biol. Chem. 280:29060–29066 (2005)). Inhibition of CamKK2 with STO609 abolished the J147 effect on AMPK activation (FIGS. 15C, 15D), indicating this is the indeed case. Although it is not clear how inhibition or knockdown of ATP synthase leads to compartmental changes in Ca²⁺ levels, it is not surprising given the role of ATP synthase in mPT pore structure and permeability, as well as the fact that Ca²⁺ flux from the mitochondria relies on H⁺ pumping by ATP synthase. Collectively, these data argue that modulation of Ca²⁺ flux is involved in the J147 and ATP synthase knockdown-mediated protection against acute toxicity as well as the activation of AMPK. These results also explain the paradoxical finding that J147 activates AMPK despite no drop in ATP levels.

AMPK selectively inhibits the mTOR pathway by preventing activation of several upstream mTOR activators, and interfering with key mTOR substrates. In particular, the ribosomal protein S6 kinase and ACC1 are two targets responsible for translation initiation and fatty acid oxidation, respectively. AMPK has been shown to decrease the phosphorylation of S6 kinase substrates, including the eukaryotic translation initiation factor 4B (eIF4B) and other effectors of translation initiation and mRNA processing. On the other hand, its phosphorylation of ACC1 prevents malonyl-CoA synthesis, thereby allowing fatty acid uptake by the mitochondria and subsequent oxidation (Grahame Hardie, J. Intern. Med. 276:543–559 (2014)). Thus, AMPK is considered to be an energy sensor inhibiting anabolic processes that consume energy, and promoting catabolic processes that produce energy (Inoki et al., Annu. Rev. Pharmacol. Toxicol. 52:381–400 (2012)). Therefore, it was determined if the aforementioned pathways were affected by J147 treatment. Western blot analysis on hippocampal lysates from J147-treated SAMP8 mice indicated that AMPK is activated in vivo, along with phosphorylation of its substrate ACC1 (FIGS. 18A, 18B). Similarly, J147 activated AMPK/mTOR signaling in vitro (FIG. 15), increasing phosphorylation of the downstream targets Raptor, S6, and ACC1. Importantly, modulation of ATP synthase activity via siRNA-targeted knockdown in MC65 cells recapitulated the J147 effect on AMPK/mTOR targets (FIG. 16), indicating that J147-mediated neuroprotection elicited by targeting ATP synthase may be due to its role in regulating both metabolism and aging.

Since modulation of AMPK/mTOR and ATP synthase can affect life span (Sun et al., *Cell Rep.* 8:1781–92 (2014)), it was determined whether J147 affects aging itself in SAMP8 mice using transcriptional drift analysis that detects age-associated decline at the molecular level. J147 treatment stabilized the hippocampal transcriptome as well as the plasma metabolome against age-associated increases in drift-variance, suggesting a younger transcriptome and metabolome.

Stabilization was most profound on carnitine metabolism and ATP synthesis coupled protein transport. Indeed, increased levels of plasma acylcarnitines in old SAMP8 mice were

detected, indicating dysfunctional mitochondrial β -oxidation of fatty acids and the pathophysiology of insulin resistance (Mihalik *et al.*, *Obesity* 18:1695–1700 (2010)). J147 not only reduced these levels in aged mice, but also restored them to levels found in young controls, consistent with drift analysis supporting J147-mediated stabilization of these processes against age. This may provide a connection to the *in vitro* effects of J147 and ATP synthase knockdown on mitochondrial metabolism. The increase in ATP and NAD⁺ in normal conditions and their preservation in our *in vitro* ischemia assay (FIGS. 13H, 13I) may be a reflection of increased ETC activity via sustained fatty acid β -oxidation. This may also explain J147-attenuation of age-associated genetic drift and longevity extension in flies as ATP and NAD⁺ are key metabolites shown to preserve metabolic integrity, and promote neuroprotection, survival, and longevity extension (Yang *et al.*, *Cell* 130:1095–1107 (2007)). Thus, targeting the mitochondria via ATP synthase stabilizes ATP synthesis during aging, resulting in the preservation of youthful molecular physiology at both the transcript and metabolite level.

Studies have demonstrated a role for ATP synthase in promoting life span extension in worms and flies via inhibition of mTOR signaling (Chin *et al.*, *Nature* 510:397-401, 2014; Sun *et al.*, *Cell Rep.* 8:1781–92, 2014). It was therefore determined if J147 could extend lifespan in *Drosophila*. It was observed that J147 extended lifespan up to 12.8%, supporting the existence of a relationship between aging and mitochondrial control of metabolism (FIG. 18F). Because J147 was identified based on its ability to protect cells from age-associated neurotoxicities *in vitro*, and reverses cognitive deficits and enhances memory in several AD mouse models, these results indicate that aging and age-associated dementia are much more closely related than previously assumed and share common drug targets.

In summary, ATP synthase was identified as a target of J147. The mitochondrial effects of J147 as well as the signaling pathways affected by modulation of ATP synthase activity are provided. A role for ATP synthase regulation of mitochondrial metabolism in cell survival against acute toxicities, and a role in modulating the aging process, are provided. The results show that a phenotypic screening approach focusing on age-associated toxicities identifies compounds such as J147 that not only reduce effects of age-associated dementia, but also provide unexpected drug targets and new molecular insights into neurodegenerative diseases such as AD by studying their mechanism of action.

Example 17

Effect of CAD031 on Aging

As described above, the effect of J147 on SAMP8 mice was investigated using an integrative multiomics approach (see FIGS. 9A-9C). These data identified a subset of metabolic changes associated with aging that may be relevant to sporadic AD and other forms of dementia. Aging progressively destabilizes transcriptomes and metabolomes causing levels of transcripts and metabolites to progressively drift away from the levels observed in young animals. This drift can be measured and has been used to determine the relative age of worms, mice, and humans, with an excellent correlation with chronological age. When this analysis was applied to gene expression data from SAMP8 mice, it demonstrated that the gene expression profile of old SAMP8 mice treated with J147 looked more like that of younger mice, again suggesting that J147 slows aging. J147 reduced cognitive deficits in old SAMP8 mice, while restoring multiple molecular markers associated with human AD, vascular pathology, impaired synaptic function, and inflammation to those approaching the young phenotype. In addition, the molecular target of J147 was identified as one of the subunits of the mitochondrial ATP synthase. This is the first identification at the molecular level of a connection between aging and old age-associated neurodegenerative disease.

The data in FIGS. 7A-7E performed with both plasma and brain samples from the same animals shows that a number of aspects of small molecule physiology that are altered with aging are maintained at a youthful level by J147. This observation is supported by drift analysis of the metabolites performed in a similar way as with the RNAseq data (FIGS. 20A-20C).

The direct effect of J147 and its derivative CAD031 in longevity is shown in FIGS. 18F and 21. J147 significantly increased the lifespan of *Drosophila* (FIG. 18F); and CAD031, a synthetic derivative of J147, dramatically extends the lifespan of SAMP8 mice when fed at 200 ppm (~10mg/kg/day) starting at 8 months of age, very old for this short-lived strain (FIG. 21). There is a median lifespan extension of nearly 40%. CAD031 is a slightly more stable version of J147 (a substitution of a methoxy group with trifluoromethoxy) and has the same potency and molecular target as J147. Thus, even though the drug was given to the mice when they were quite old, it still significantly extended lifespan. Thus, J147 and derivative molecules thereof can be used to prevent aspects of aging.

Example 18

Renoprotective effects of J147 and CMS121

This example describes methods used to demonstrate the effect of J147 and CMS-121 on chronic kidney disease and diabetic kidney disease.

A number of pathogenic mechanisms are linked to both chronic kidney disease and diabetic nephropathy, including oxidative stress, inflammation and mitochondrial dysfunction (Calcutt *et al.*, *Nat Rev Drug Discov* 8:417-429, 2009). Curcumin exhibits protective effects in rodent models of diabetic nephropathy (Ho *et al.*, *Am J Med Sci* 351:286-295, 2016). The ability of the synthetic derivative of curcumin J147, to have renoprotective effects was determined by measuring kidney weight and expression of TNF- α , 12-lipoxygenase (12-Lox) and caspase 1 as indices of inflammation and cell disruption in the SAMP8 mouse model of chronic kidney disease associated with rapid aging and SAMP8 mice treated with J147 for three months starting at 9 months of age. J147 was given at 10/mg/kg/day. This is a very old age for these mice because they only live for 14-15 months and have serious cognitive and physiological damage at 8 months (Currais *et al.*, *Aging* (Albany NY) 7:937-955, 2015).

As shown in FIGS. 22A-22D, kidney weight decreased (body weight did not) between 9-12 months of age and this loss of weight was accompanied by increased expression of TNF α , 12-Lox and cleaved caspase 1 protein. All of these aging-related changes were prevented or significantly attenuated by J147 treatment (FIGS. 22A-22D). These data demonstrate the renoprotective effects of J147. Thus, J147 can be used to prevent or reverse indices of diabetic nephropathy.

In another experiment, mice were treated with CMS-121 at 200 ppm (about 10/mg/kg/day), in the same manner as described for J147. As shown in FIGS. 23A-23F, the protective response with CMS-121 was better than J147. Table 3 shows the structure of CMS-121 and other fisetin derivatives that can be used to treat or prevent kidney diseases, such as those associated with aging.

Table 3: Fisetin and derivatives thereof

Comp No	Structure
fisetin	
geraldol	
04P	
011	
023	
024	
034	
121	
140	

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In view of the many possible embodiments to which the principles of the disclosure may be applied, it should be recognized that the illustrated embodiments are only examples of the disclosure and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

20

We claim:

1. A method of identifying an agent to treat or prevent a neurodegenerative disease, comprising:

5 contacting one or more test agents with one or more components of the mitochondrial permeability transition pore (MPTP);

assaying for 5' AMP-activated protein kinase (AMPK) activity, mitochondrial permeability transition pore (MPTP) amount or activity, ATPsyn activity or expression, ATPase activity, ATP production, NAD⁺ production, NADH production, NADPH production, mitochondrial calcium
10 levels, or combinations thereof; and

selecting the one or more test agents that alter the AMPK activity, MPTP amount or activity, ATPsyn activity or expression, ATPase activity, ATP production, NAD⁺ production, NADH production, NADPH production, mitochondrial calcium levels by at least 10% as compared to the AMPK activity, MPTP amount or activity, ATPsyn activity or expression, ATPase activity,
15 ATP production, NAD⁺ production, NADH production, NADPH production, mitochondrial calcium levels in an absence of the one or more test agents.

2. The method of claim 1, wherein the one or more components of the MPTP comprises a mitochondrial ATP synthase (ATPsyn).
20

3. The method of claim 1, wherein the one or more components of the MPTP comprises a mitochondrial ATP synthase alpha subunit.

4. The method of claim 3, wherein the mitochondrial ATP synthase alpha subunit comprises at
25 least 80%, at least 90%, at least 95%, or at least 99% sequence identity to the amino acid sequence of GenBank Accession No. BAA03531.1, CAA46452.1, or NP_075581.1.

5. The method of claim 1, wherein the one or more components of the MPTP comprises a J147 binding site.
30

6. The method of claim 1, further comprising:

administering the selected one or more test agents to a non-human mammal;

assaying for AMPK activity, MPTP amount or activity, ATPsyn activity or expression, ATPase activity, ATP production, NAD⁺ production, NADH production, NADPH production, mitochondrial calcium levels, or combinations thereof; and

selecting the one or more test agents that alter the AMPK activity, MPTP amount or activity, ATPsyn activity or expression, ATPase activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels by at least 10% as compared to the AMPK activity, MPTP amount or activity, ATPsyn activity or expression, ATPase activity, ATP production, NAD⁺ production, NADH production, NADPH production, and/or mitochondrial calcium levels in an absence of the one or more test agents.

7. The method of claim 6, wherein the non-human mammal is a rat, mouse, or non-human primate.

8. The method of any of claims 1-5, wherein the one or more components of the MPTP are present in a cell, and wherein contacting comprises contacting the one or more test agents with the cell.

9. The method of claim 8, wherein the cell is a neural cell.

10. The method of claim 9, wherein the neural cell is a hippocampal cell.

11. A method of treating or preventing a neurodegenerative disease in a subject; comprising:
administering a therapeutic amount of an agent that modulates AMPK activity, MPTP amount or activity, ATPsyn activity or expression, ATPase activity, ATP production, NAD⁺ production, NADH production, NADPH production, mitochondrial calcium levels, or combinations thereof in a neural cell of the subject.

12. The method of claim 11, wherein the agent is identified using the method of any of claims 1-10.

13. The method of any of claims 11-12, further comprising administering a therapeutic amount of an additional agent that can treat or prevent a neurodegenerative disease.

14. The method of any of claims 11-13, wherein the agent increases AMPK activity, MPTP amount or activity, ATPsyn activity or expression, ATPase activity, ATP production, NAD⁺

production, NADH production, NADPH production, mitochondrial calcium levels, or combinations thereof.

15. The method of any of claims 1 to 14, wherein the neurodegenerative disease is Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), glaucoma, retinal degeneration, macular degeneration, age-related hearing loss, mild cognitive impairment, dementia (including, for example, frontotemporal dementia, AIDS dementia, and the like), progressive supranuclear palsy, and/or spinocerebellar ataxias.

16. A method of treating or preventing one or more signs of aging in a subject; comprising:
administering a therapeutic amount of one or more compounds disclosed herein to the subject.

17. A method of treating or preventing a kidney disease in a subject; comprising:
administering a therapeutic amount of one or more compounds disclosed herein to the subject.

18. The method of claim 17, wherein the kidney disease is chronic kidney disease and diabetic neuropathy.

19. The method of any of claims 16 to 18, further comprising administering a therapeutic amount of an additional agent that can treat or prevent one or more signs of aging or kidney disease.

20. The method of any of claims 11 to 19, wherein the subject is a mammal.

21. The method of any of claims 16 to 20, wherein the one or more compounds decreases cytoskeleton-associated protein (Arc), decreases synapse-associated protein 102 (SAP102), decreases phosphorylation of eukaryotic initiation factor 2a (eIF2 α), increases the amount of eIF2 α , decreases the amount of heat shock protein 60 (HSP60), increases the amount of HSP90, decreases the amount of amyloid precursor protein (APP), decreases the amount of APP fragment C99, decreases the amount of APP fragment C83, decreases the level of A β ₁₋₄₀S, decreases the level of total tau protein, decreases levels of tau protein phosphorylation at Ser396, decreases the level of vascular cell adhesion molecule 1 (VCAM-1), decreases the level of endogenous immunoglobulin G (IgG), decreases glial fibrillary acidic protein (GFAP) expression, decreases activation of the

stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK), decreases upregulation (e.g., reduces expression) of inflammatory genes such as mitogen-activated protein kinase (MAPK) kinases, such as Map3k7 and Map2k4), increases expression of *Flt1* that encodes the vascular endothelial growth factor receptor 1, increases levels of docosahexaenoic acid (DHA), increases
 5 levels of adrenic acid, increases anti-oxidant effects (e.g., by increasing the AA-metabolites 9-HETE and 8-iso-15-keto PGF₂ β ; the DHA-metabolites 11- and 13-HDoHEs; and the LA-metabolites 9-HODE and 13-HODE), reverse levels of cytochrome P450 metabolites 19-HETE and 20-HETE (AA derivatives), 19(20)-EpDPE (DHA derivative) and 9,10-DiHOME (LA derivative) that occur with aging, reduce levels of TXB2, reverse levels of 20-HETE and 9,10-DiHOME that
 10 occur with aging, increase levels of γ -glutamyl amino acids, increase levels of branched-chain amino acids (BCAAs), increase levels of dipeptides, decrease levels of acylcarnitines, decrease levels of PUFAs, decrease levels of cAMP, reverse levels of glycolytic and TCA intermediates indicative of mitochondrial dysfunction that occur with aging, increase the level of glutamate, increase the level of α -ketoglutarate, decrease levels or expression of mTor, reverse levels of
 15 molecules listed in Tables 1 and 2 that occur with aging, or combinations thereof.

22. The method of any of claims 16 to 21, wherein the signs of aging comprises one or more of compromised BBB homeostasis, compromised brain vascular function, increased brain inflammation, a pro-oxidant status of the brain, and kidney disease.

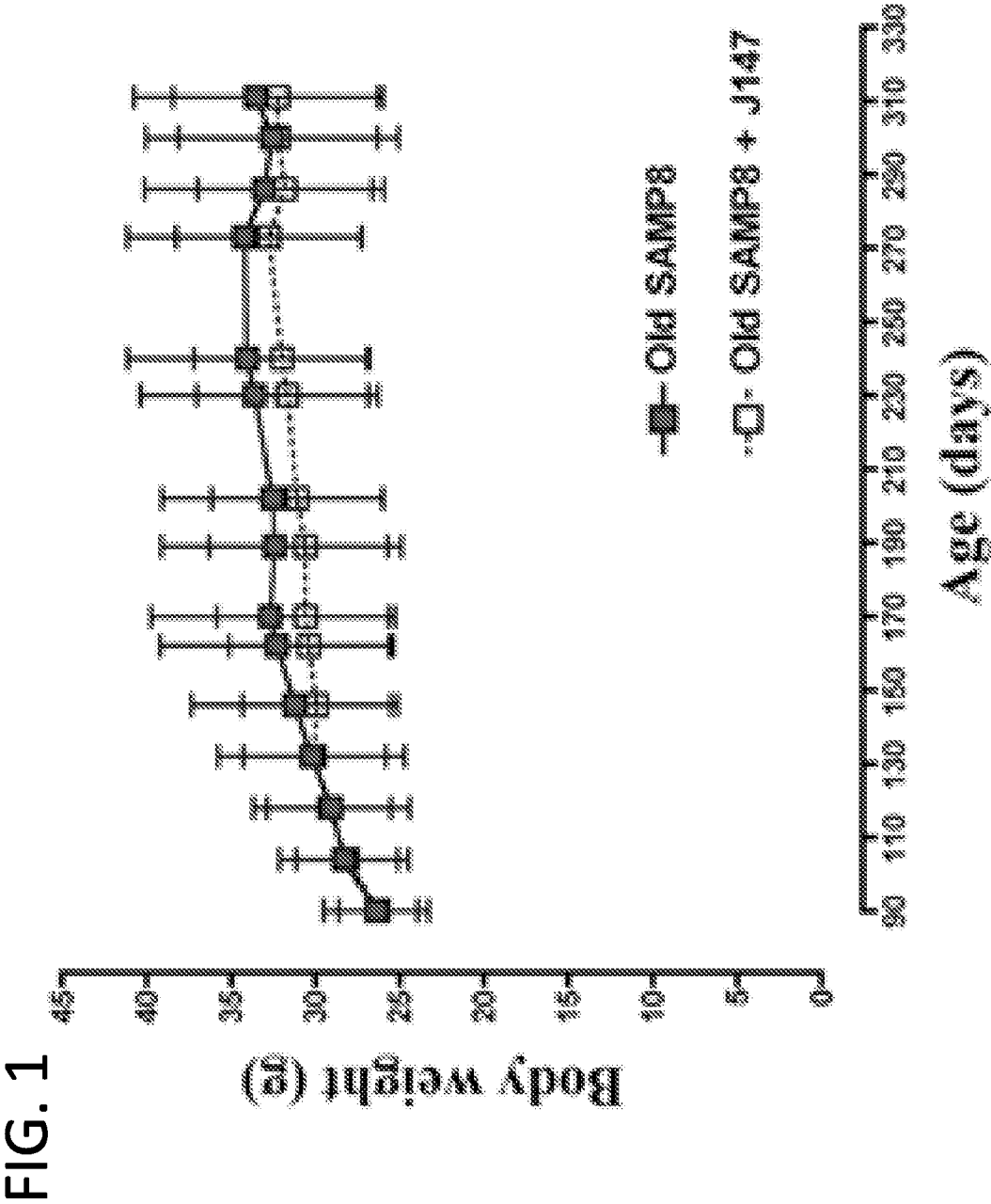
23. The method of any of claims 16 to 22, wherein the method slows down one or more signs of aging by at least 10% as compared to no administration of the one or more compounds.

24. The method of any of claims 16 to 23, wherein the method increases longevity by at least 10% as compared to no administration of the one or more compounds.

25. The method of any of claims 16 to 24, wherein the one or more compounds comprise J147 and/or CAD031.

26. The method of any of claims 16 to 25, wherein the one or more compounds increase kidney weight, decrease TNF-alpha expression, decrease 12-Lox expression, decrease cleaved caspase 1 protein, decrease p65 expression, decrease iNOS expression, or combinations thereof.

27. The method of any of claims 16 to 26, wherein the subject does not have Alzheimer's disease.



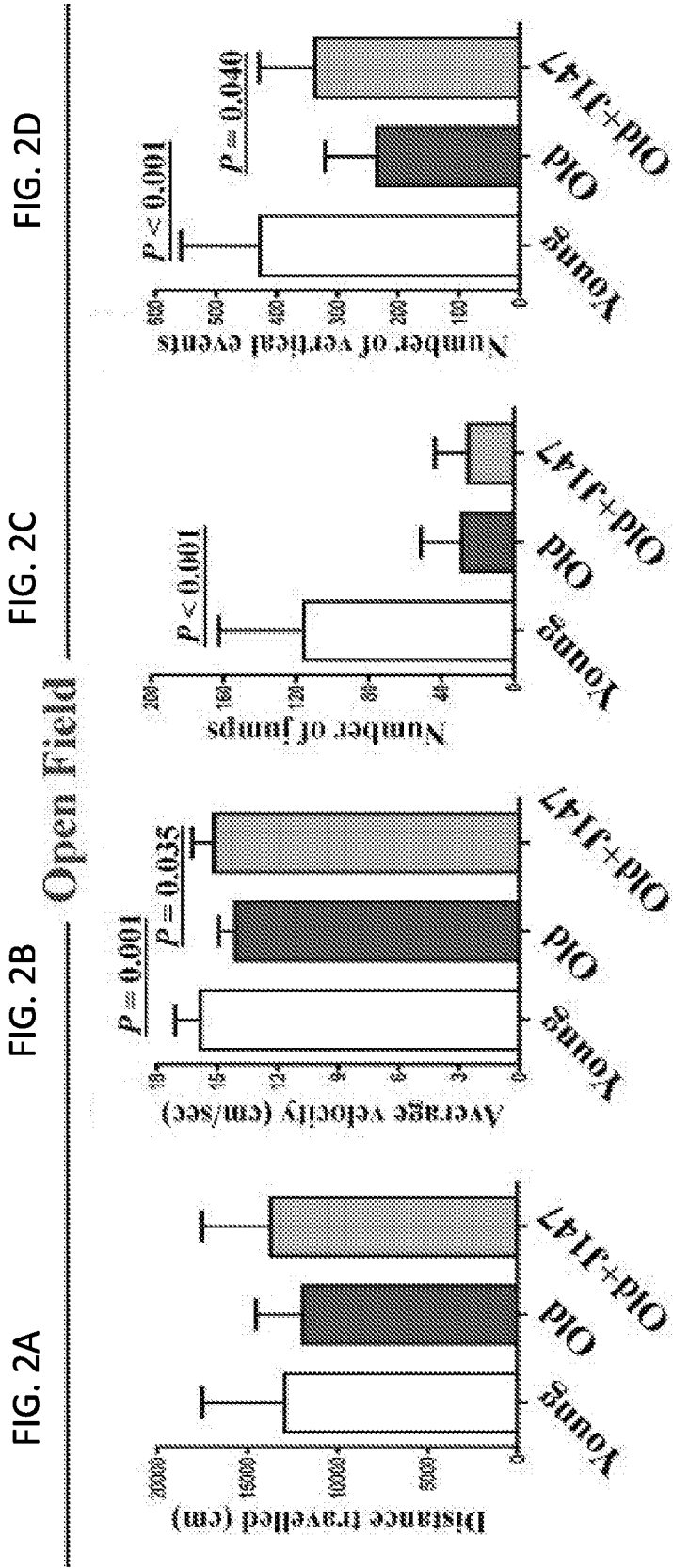


FIG. 2E

— Elevated Plus Maze —

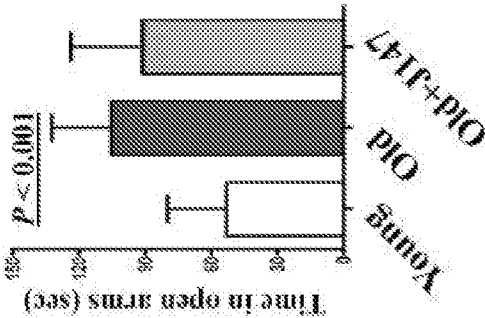


FIG. 2F

— Object Recognition —

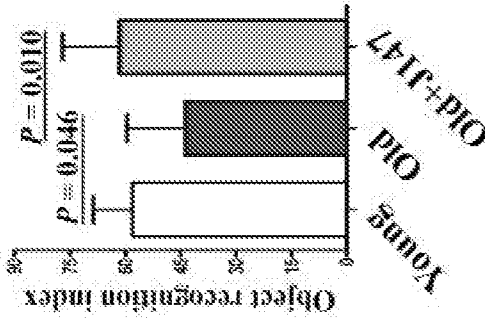


FIG. 2G

— Barnes Maze Reversal —

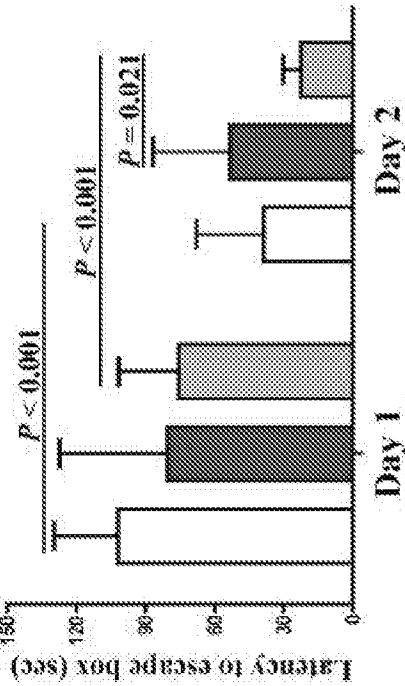


FIG. 3A
Arc/Actin

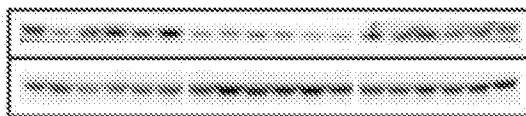
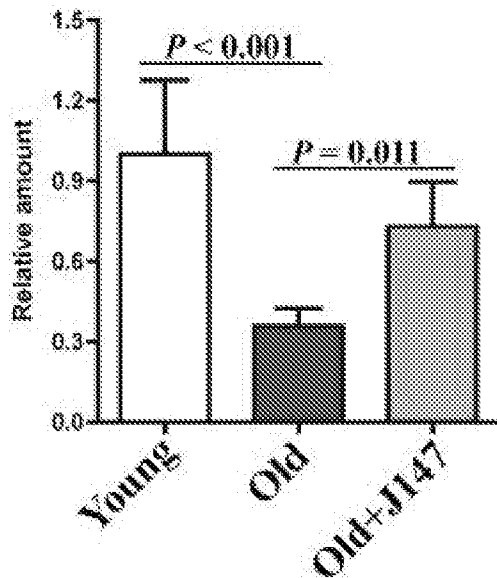


FIG. 3B
SAP102/Actin

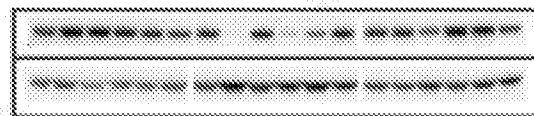
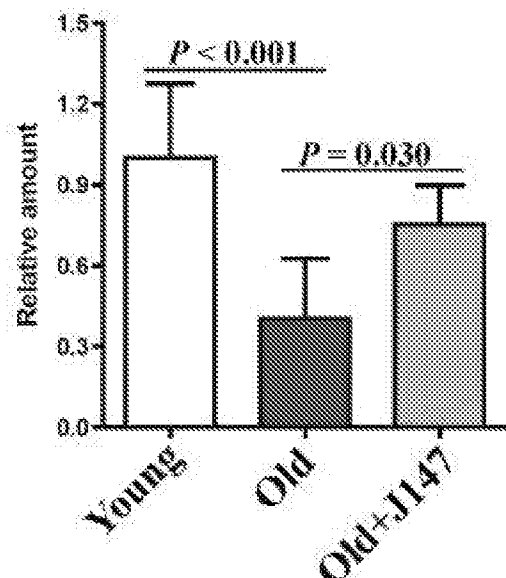


FIG. 3C
eIF2 α /Actin

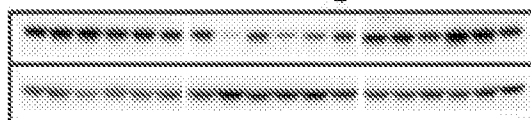
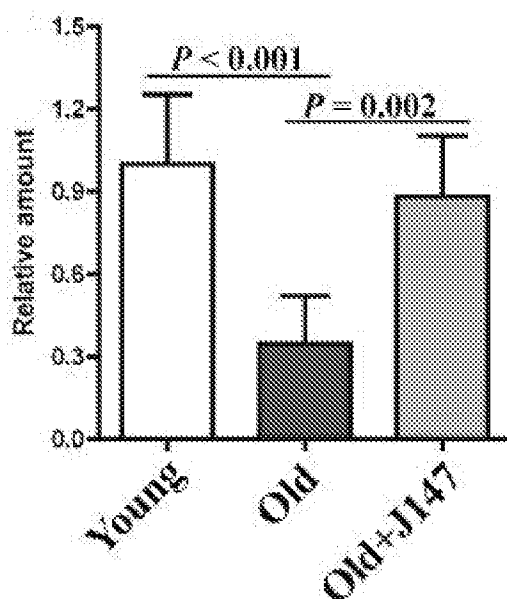
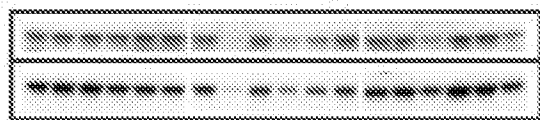
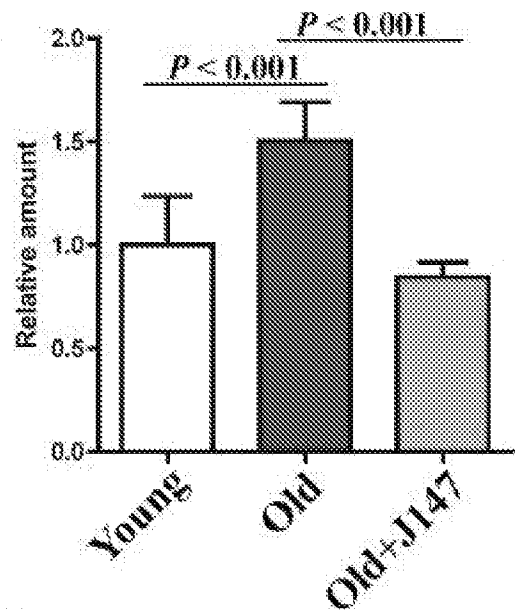


FIG. 3D
p-eIF2 α /eIF2 α



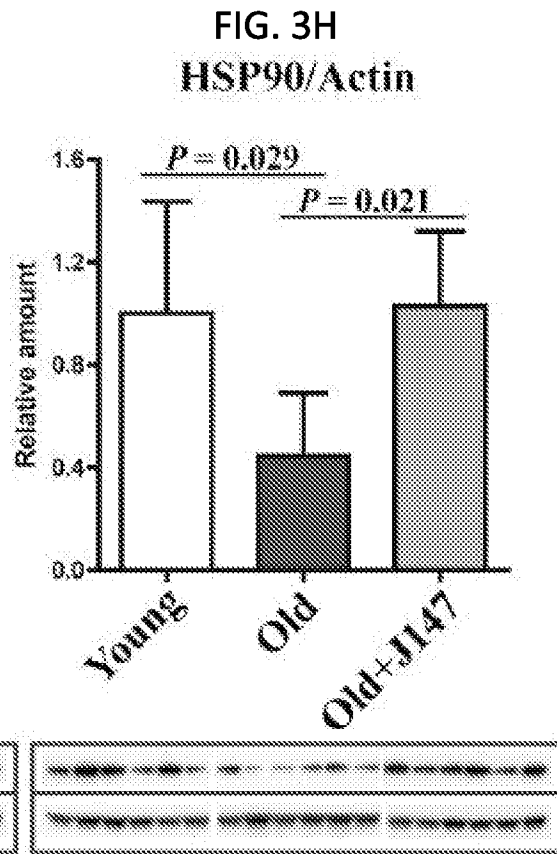
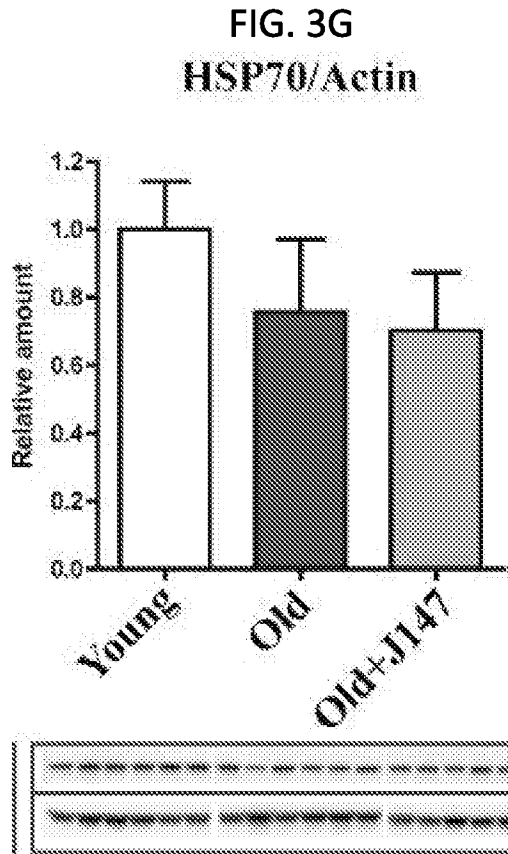
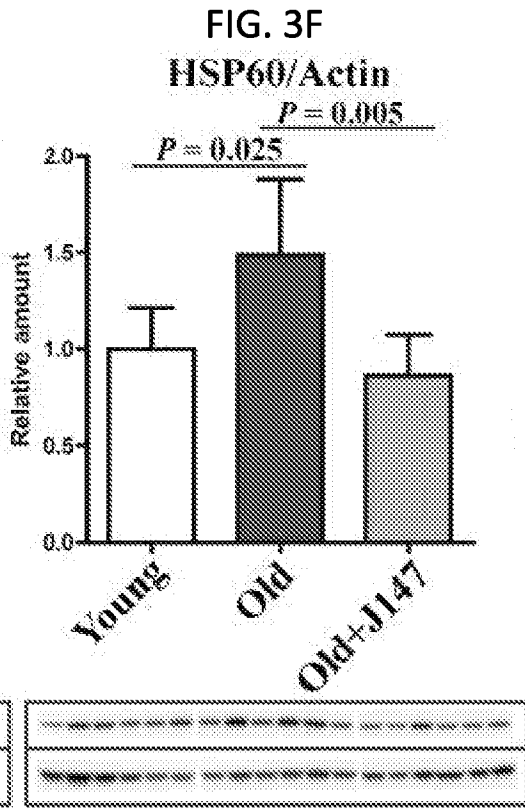
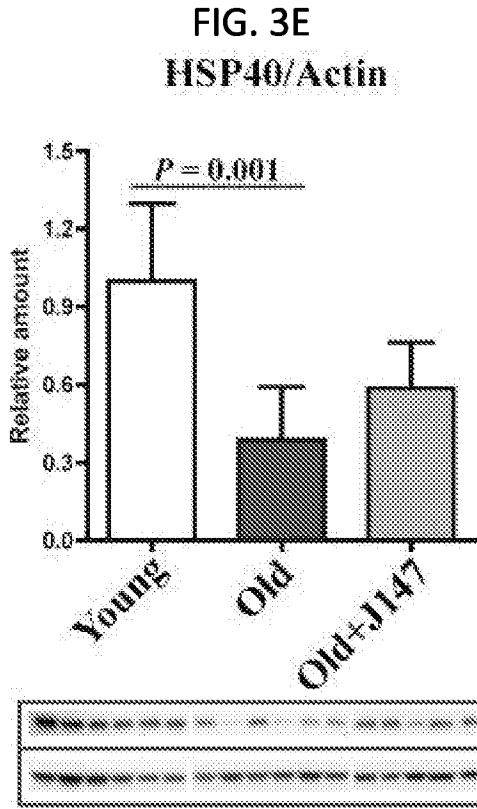


FIG. 4A

APP/Actin

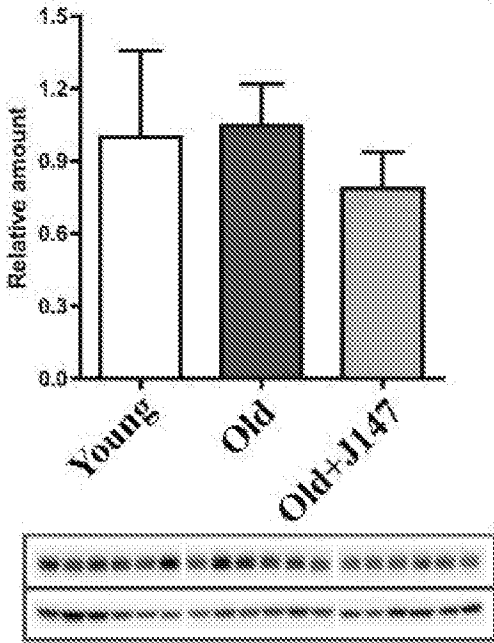


FIG. 4B

C99/APP

C83/APP

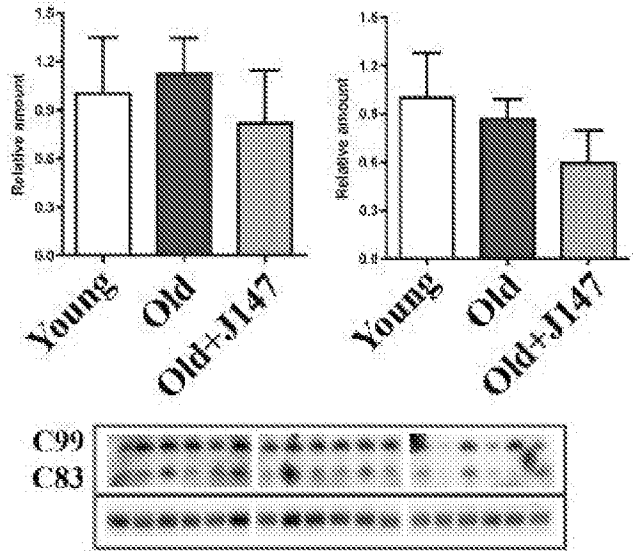
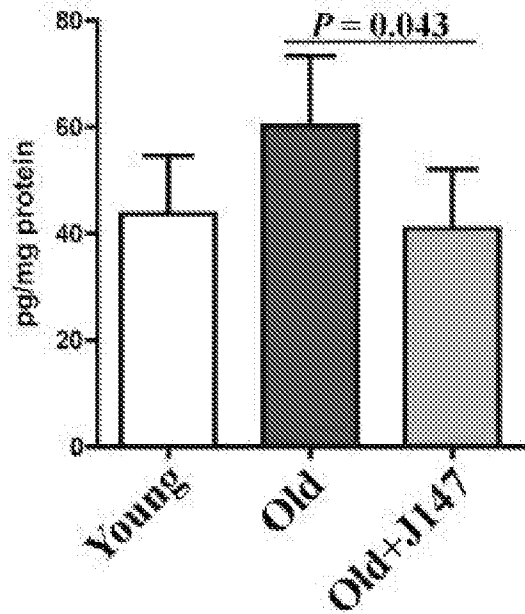


FIG. 4C

A β ₁₋₄₀ ELISA



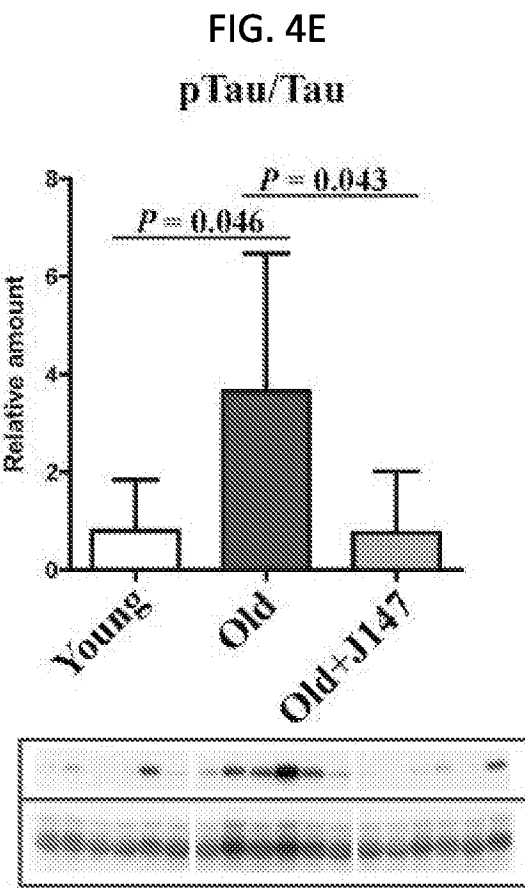
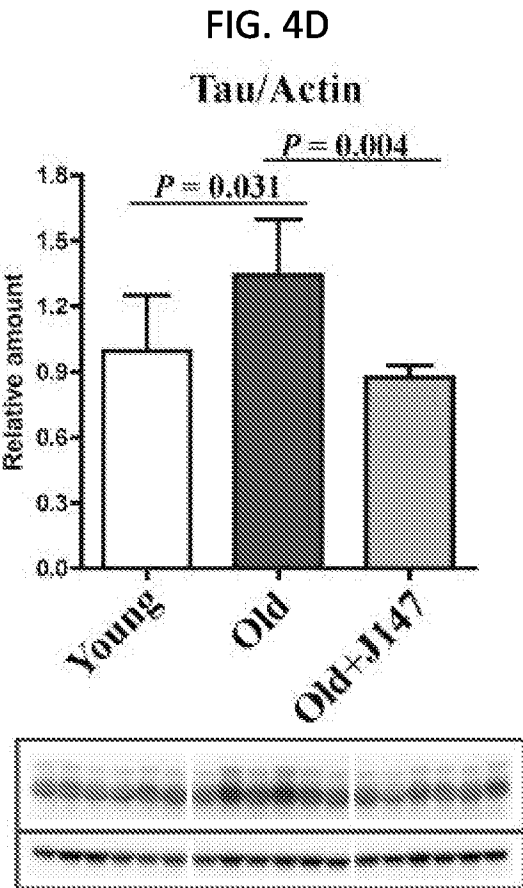


FIG. 5A

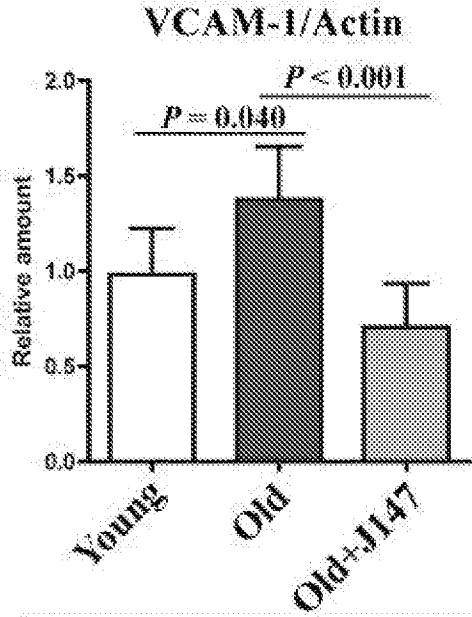


FIG. 5B

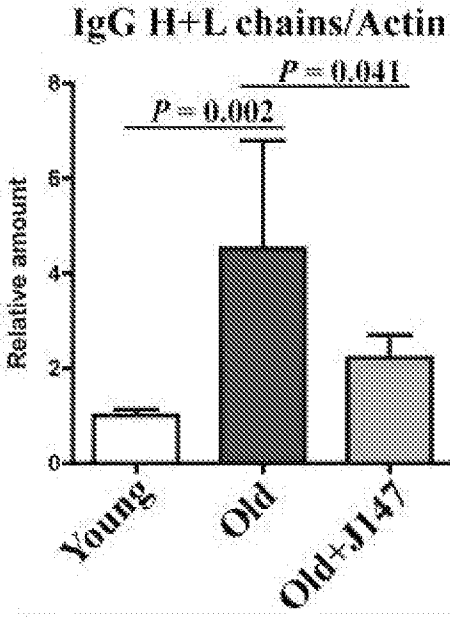


FIG. 5C

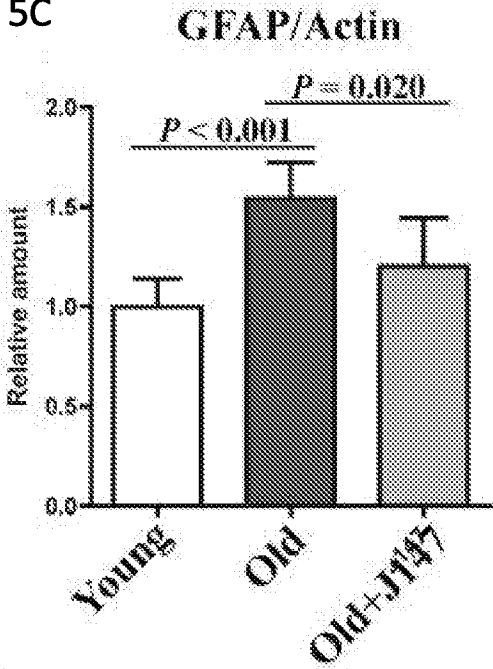


FIG. 5D

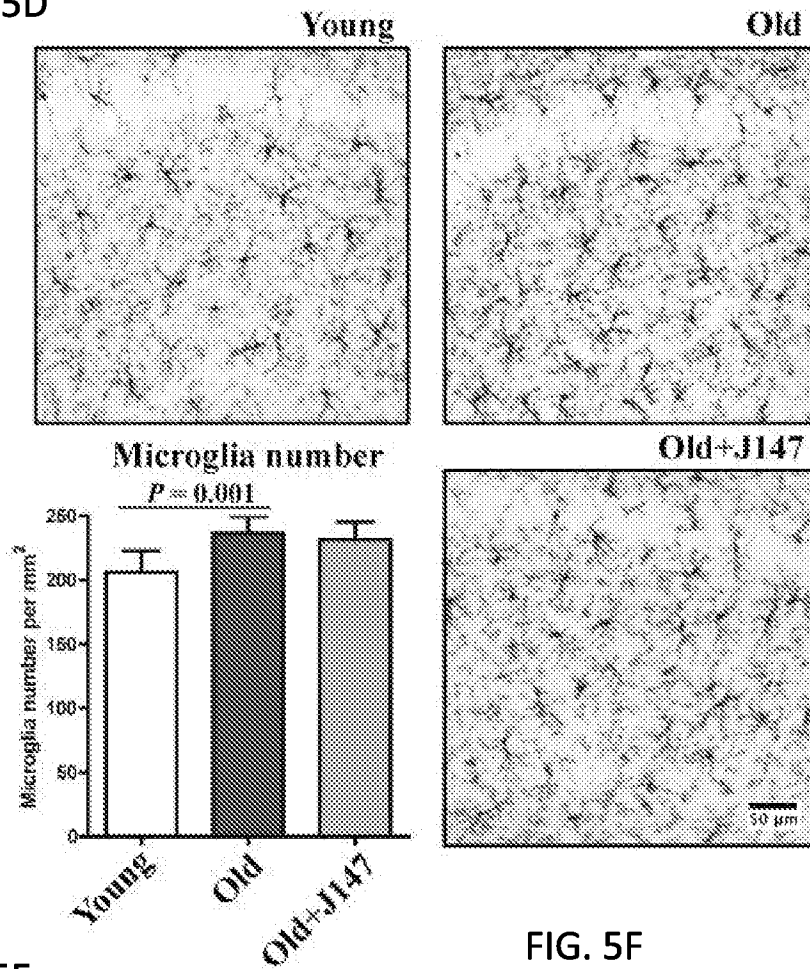


FIG. 5E

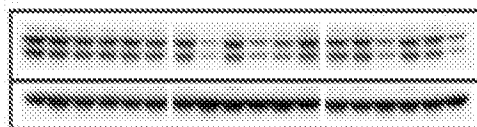
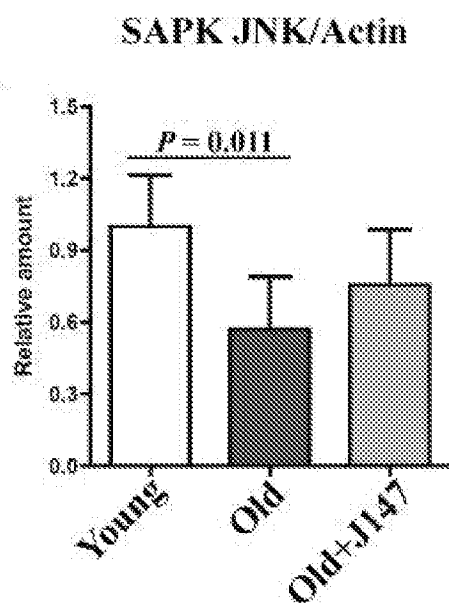
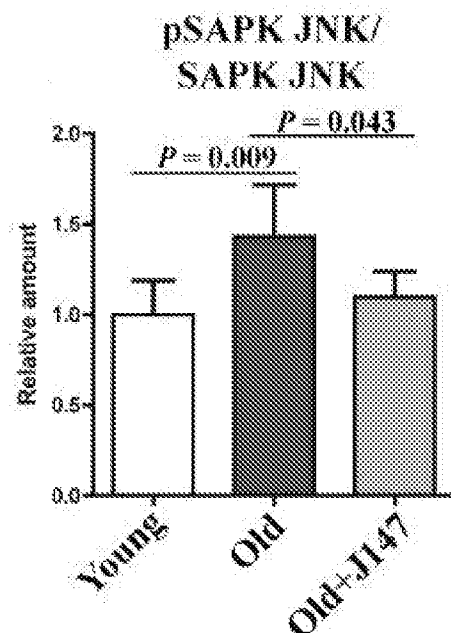


FIG. 5F



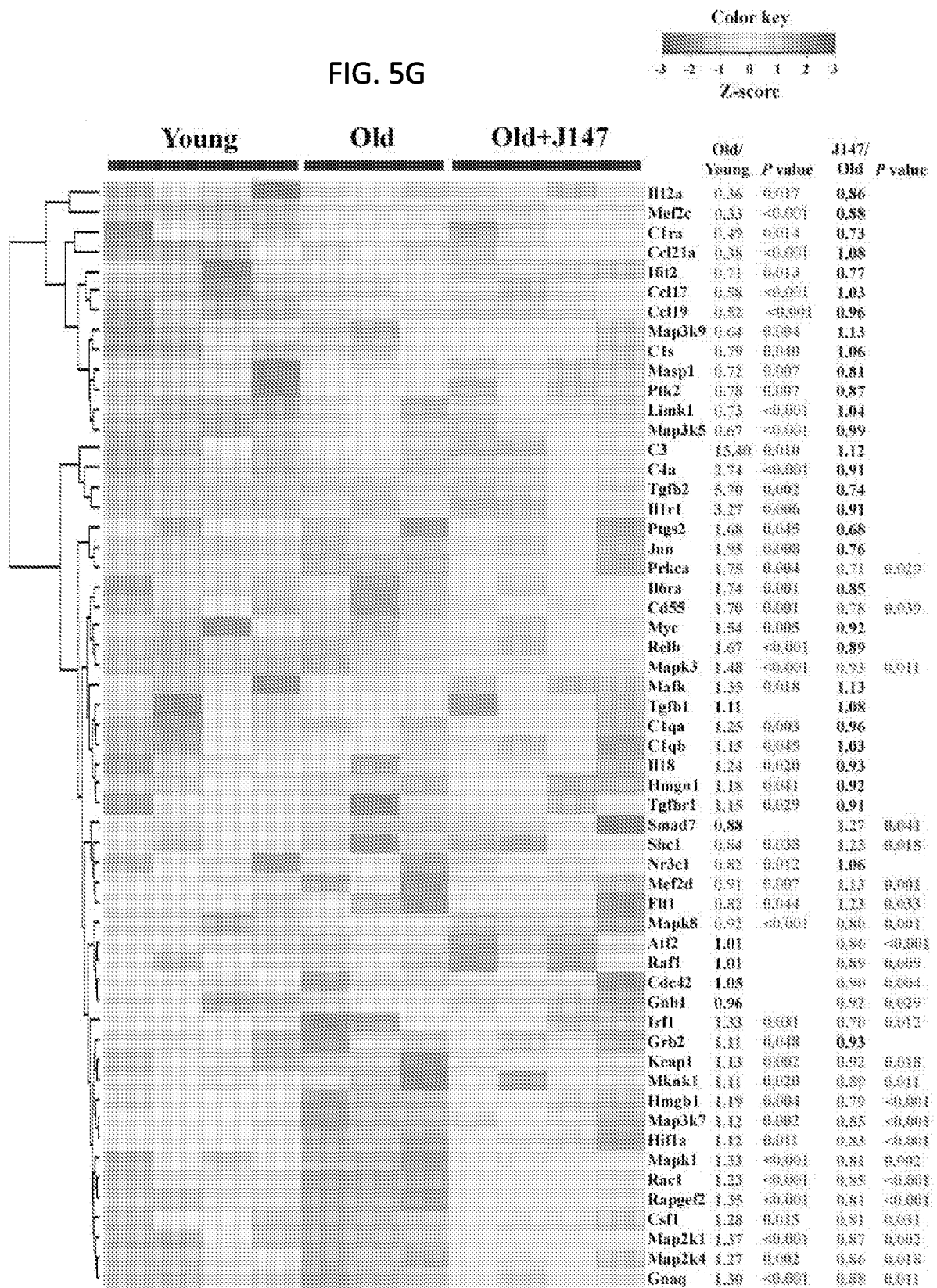
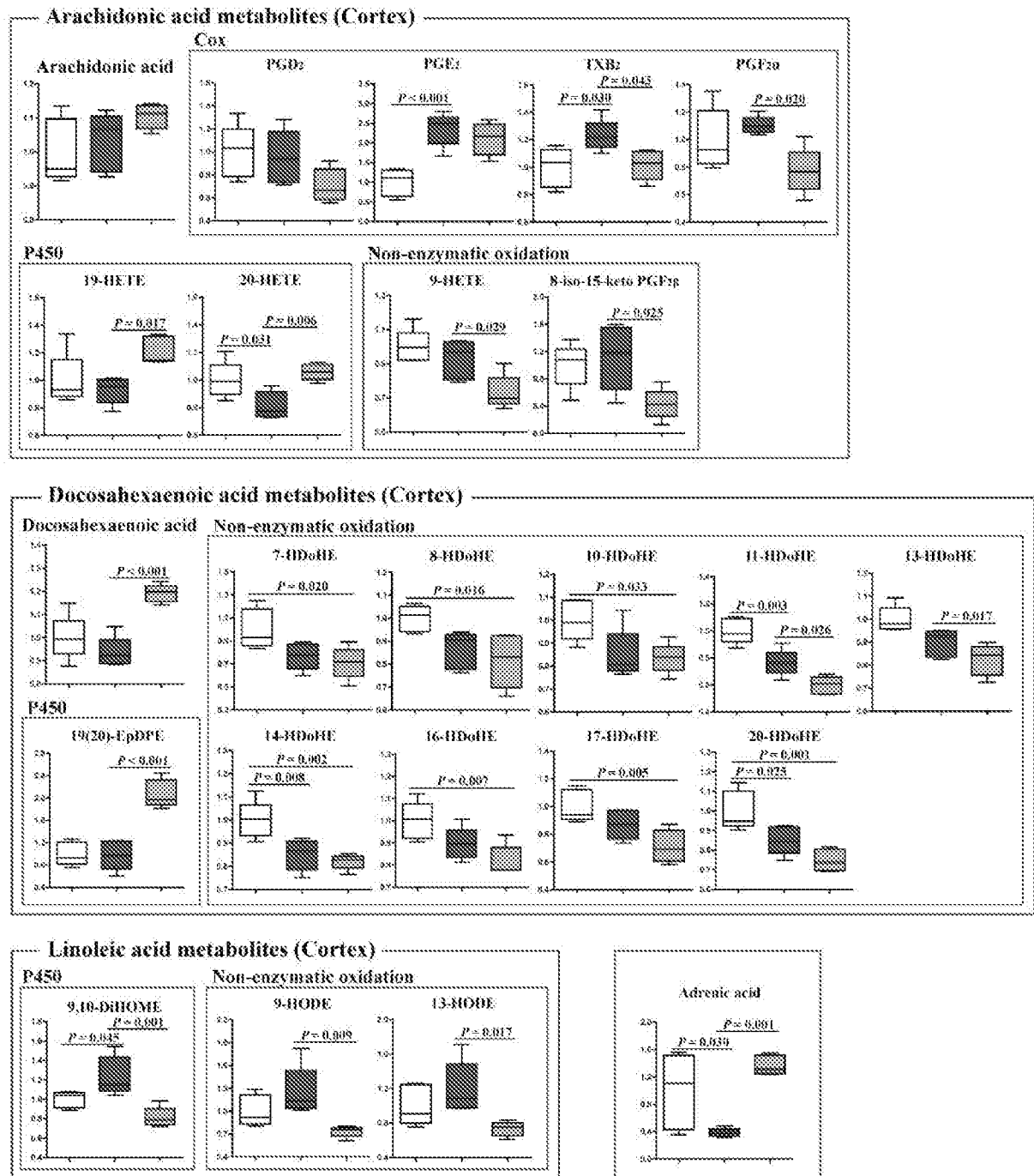
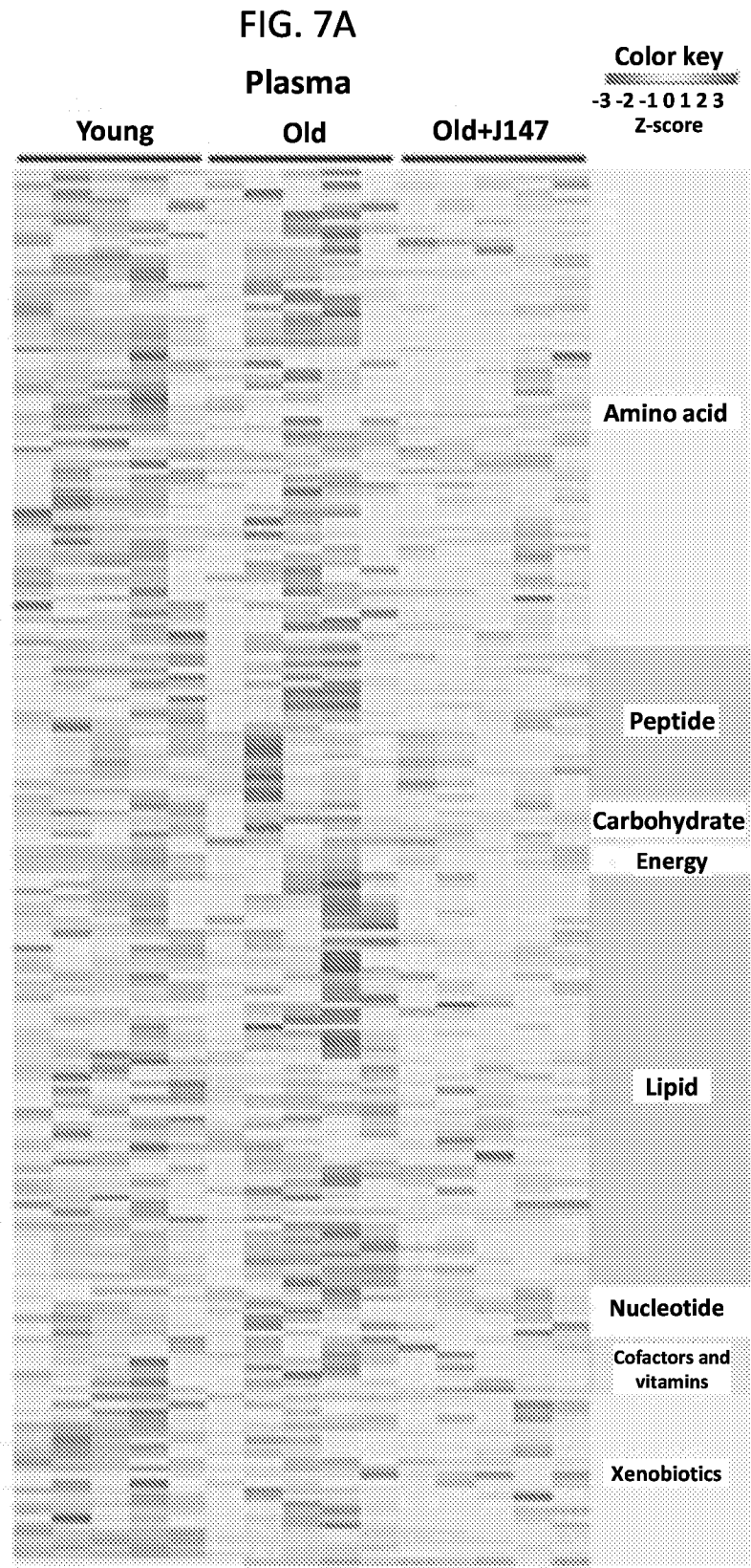


FIG. 6





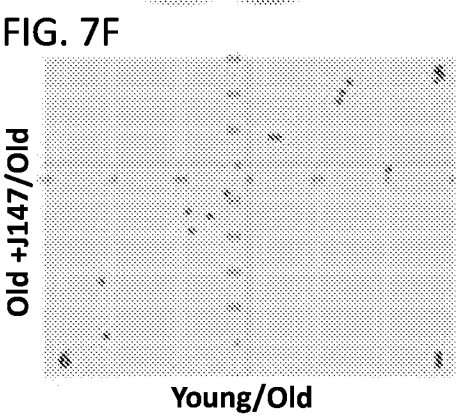
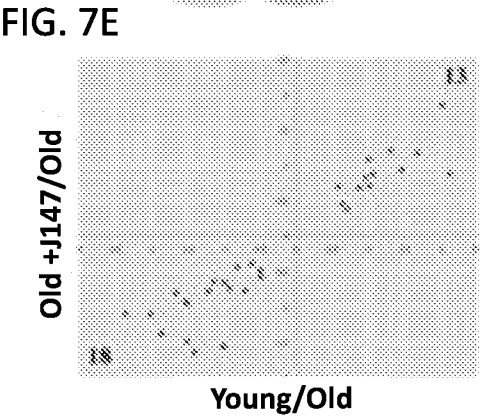
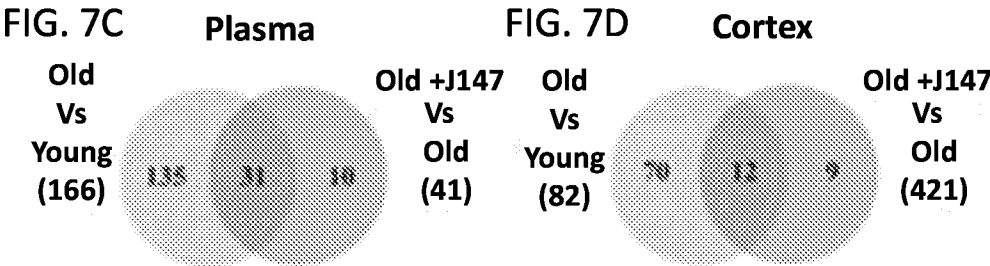
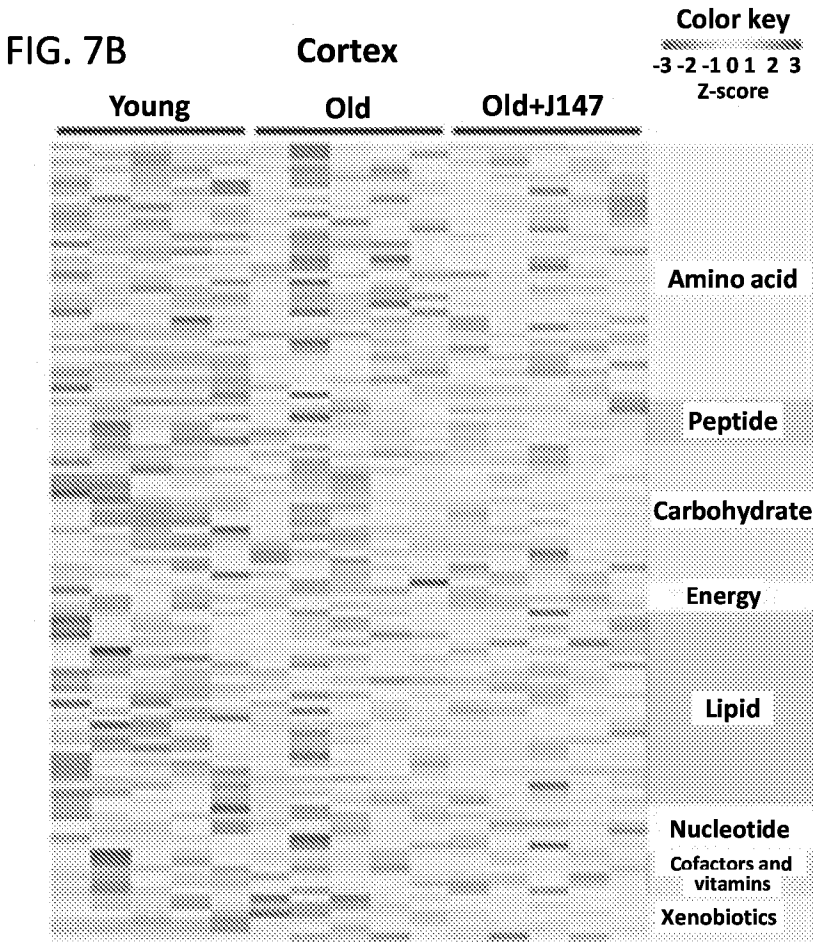


FIG. 7G

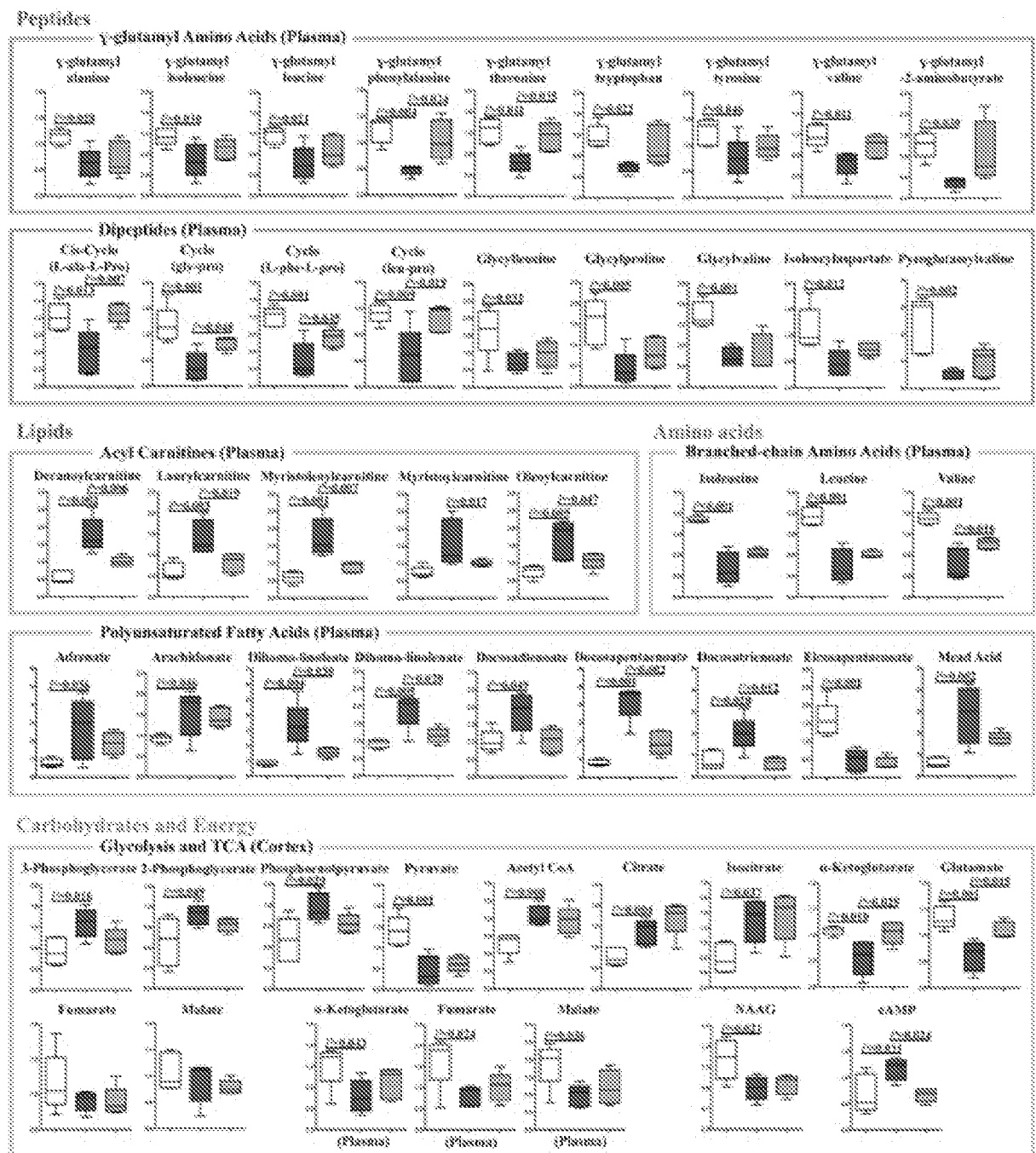


FIG. 8A

Number Biochemicals/Group Altered

Old Vs Young	
> Leucine, Isoleucine and Valine Metabolism	14/39
> Lysolipid	12/71
> Dipeptide	10/16
> Gamma-glutamyl Amino Acid	9/14
> Polyunsaturated Fatty Acid (n3 and n6)	9/14
> Phenylalanine and Tyrosine Metabolism	8/24
> Lysine Metabolism	7/11
> Tryptophan Metabolism	7/16
> Food Component/Plant	7/28
> Urea cycle, Arginine and Proline Metabolism	6/12
> Fatty Acid, Dicarboxylate	6/15
> Benzamide Metabolism	6/17
> Fatty Acid Metabolism (Acyl Carnitine)	5/14
> Methionine, Cysteine, SAM and Taurine Metabolism	5/16
> Chemical	5/19
> TCA Cycle	4/8
> Drug	2/4
> Alanine and Aspartate Metabolism	2/6
> Glycine, Serine and Threonine Metabolism	3/10
> Pentose Metabolism	3/10
> Long Chain Fatty Acid	3/15
> Riboflavin Metabolism	2/2
> Fatty Acid Metabolism (Acyl Glycine)	2/4
> Inositol Metabolism	2/4
> Pyrimidine Metabolism, Uracil containing	2/4
> Ascorbate and Aldarate Metabolism	2/4
> Purine Metabolism, Guanine containing	2/4
> Nicotinate and Nicotinamide Metabolism	2/6
	2/7

Old+J147 Vs Old

> Fatty Acid Metabolism (Acyl Carnitine)	4/14
> Polyunsaturated Fatty Acid (n3 and n6)	4/14
> Dipeptide	4/16
> Endocannabinoid	2/6
> Purine Metabolism, Guanine containing	2/6
> Pentose Metabolism	2/10
> Gamma-glutamyl Amino Acid	2/14
> Fatty Acid, Dicarboxylate	2/15
> Methionine, Cysteine, SAM and Taurine Metabolism	2/16
> Chemical	2/19
> Leucine, Isoleucine and Valine Metabolism	2/39

Plasma FIG. 8B

Canonical Pathways

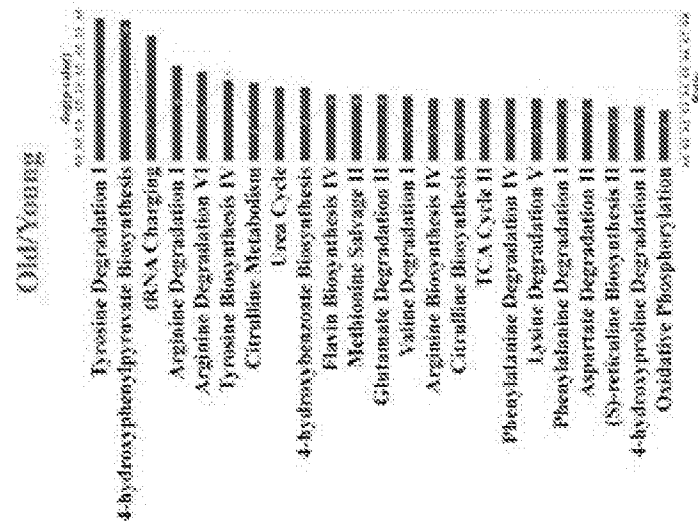


FIG. 8C

Diseases and Functions

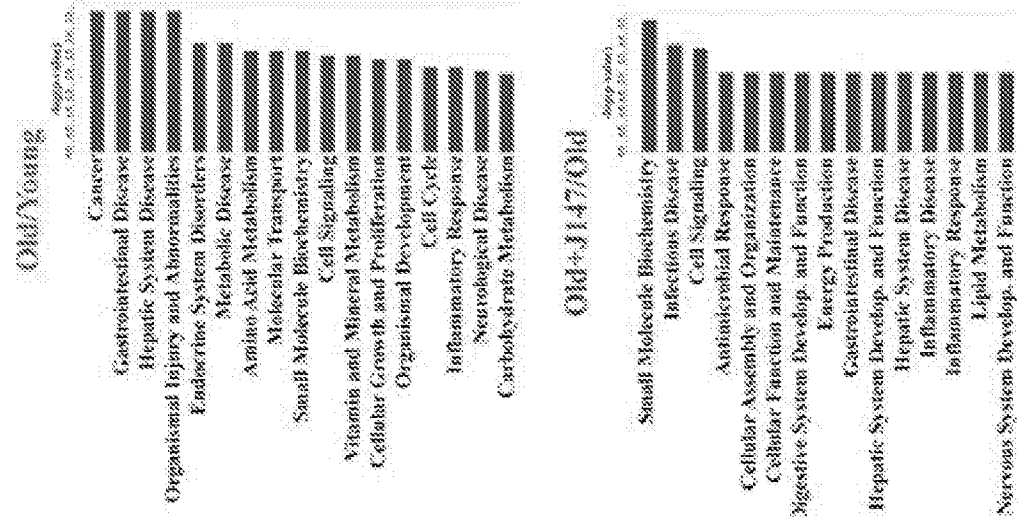


FIG. 8D

Number Biochemicals/Group Altered

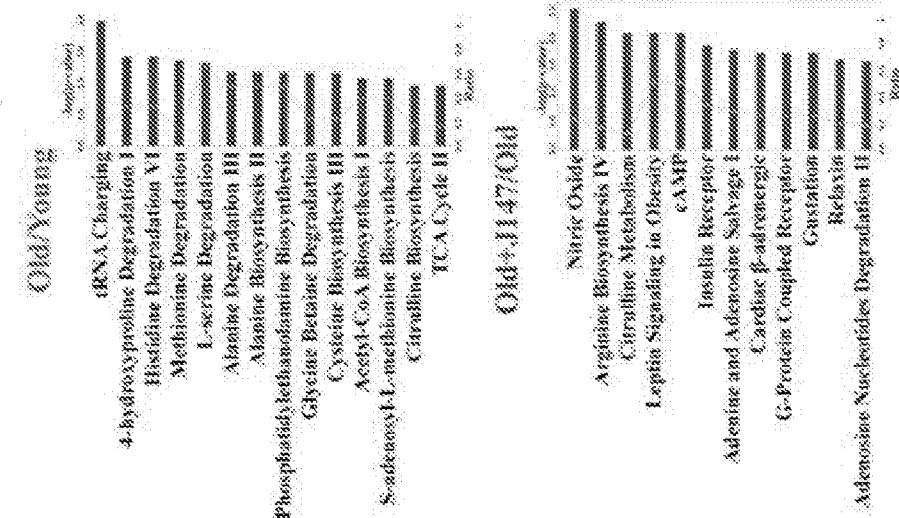
Old V's Young	
> Glutamate Metabolism	5/9
> Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	5/12
> Polyunsaturated Fatty Acid (n3 and n6)	5/13
> Methionine, Cysteine, SAM and Taurine Metabolism	5/16
> TCA Cycle	4/7
> Food Component/Fat	4/9
> Glycine, Serine and Threonine Metabolism	4/10
> Urea cycle: Arginine and Proline Metabolism	4/11
> Pantoic acid and CoA Metabolism	3/4
> Nucleotide Sugar	3/6
> Fatty Acid, Monohydroxy	3/6
> Phospholipid Metabolism	3/9
> Gamma-glutamyl Amino Acid	3/11
> Leucine, Isoleucine and Valine Metabolism	3/20
> Fatty Acid, Dicarboxylate	2/3
> Amino sugar Metabolism	2/6
> Pentose Metabolism	2/6
> Pentose Phosphate Pathway	2/6
> Lysine Metabolism	2/9
> Phenylalanine and Tyrosine Metabolism	2/10

Old+J147 V's Old

Food Component/Fat	
> Purine Metabolism, Adenine containing	3/9
> Leucine, Isoleucine and Valine Metabolism	3/14
> TCA Cycle	3/26
> Urea cycle: Arginine and Proline Metabolism	2/7
> Urea cycle: Arginine and Proline Metabolism	2/11

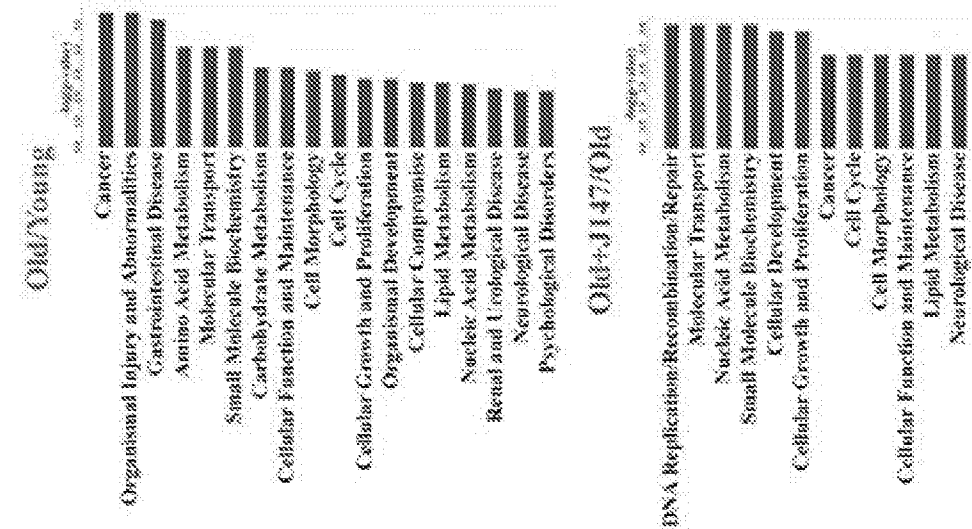
FIG. 8E
Cortex

Canonical Pathways



Old+J147/Old

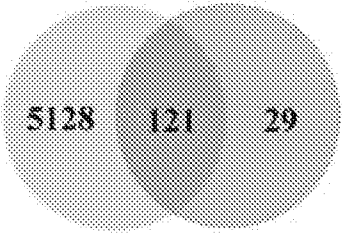
Diseases and Functions



Old+J147/Old

FIG. 9A

Old
Vs
Young
(5279)



Old + J147
Vs
Old
(150)

FIG. 9B

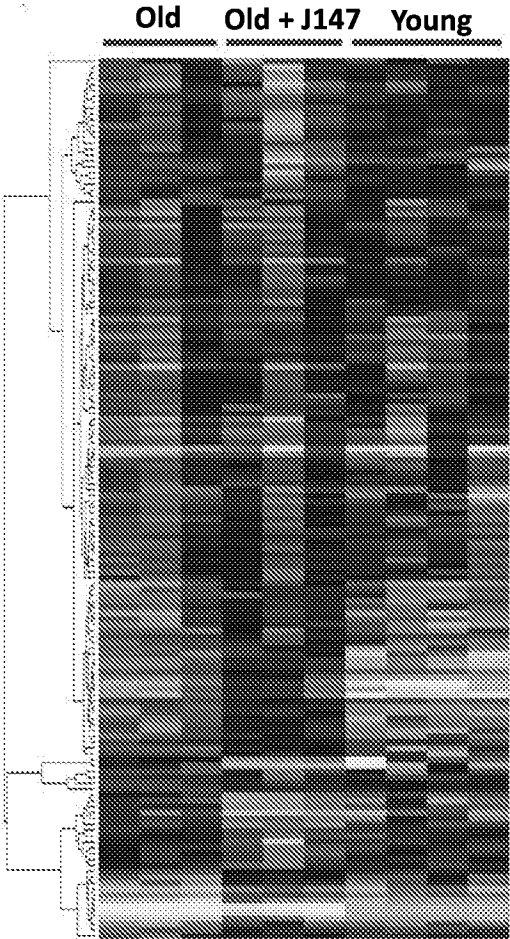


FIG. 9C

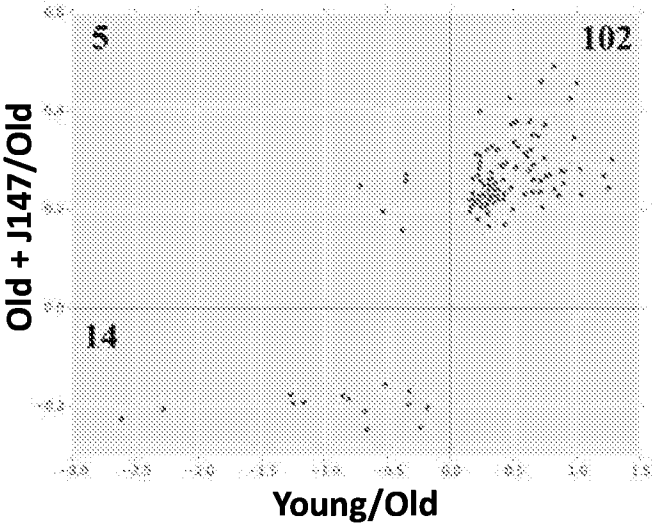


FIG. 9D
Canonical Pathways

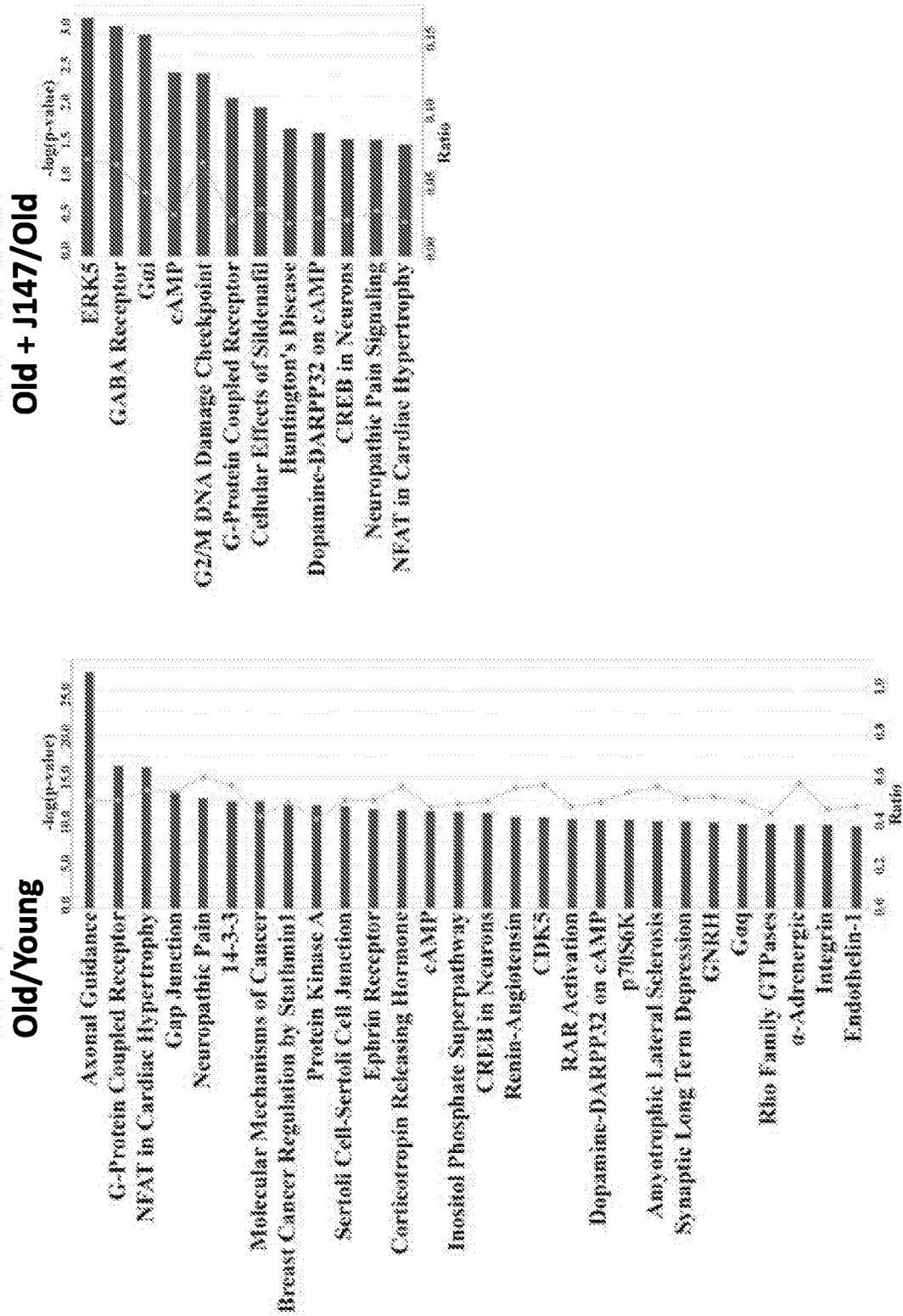
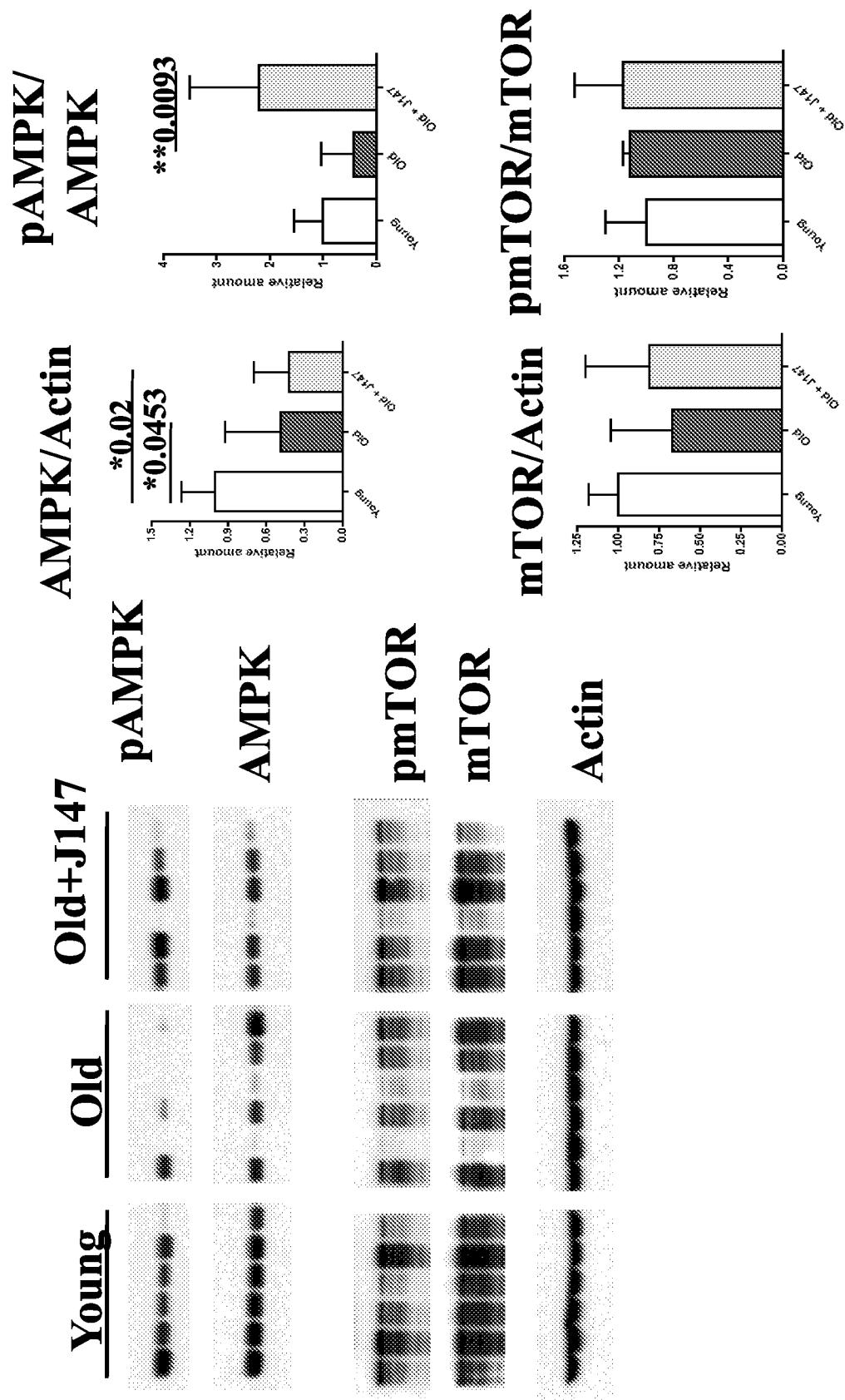


FIG. 10



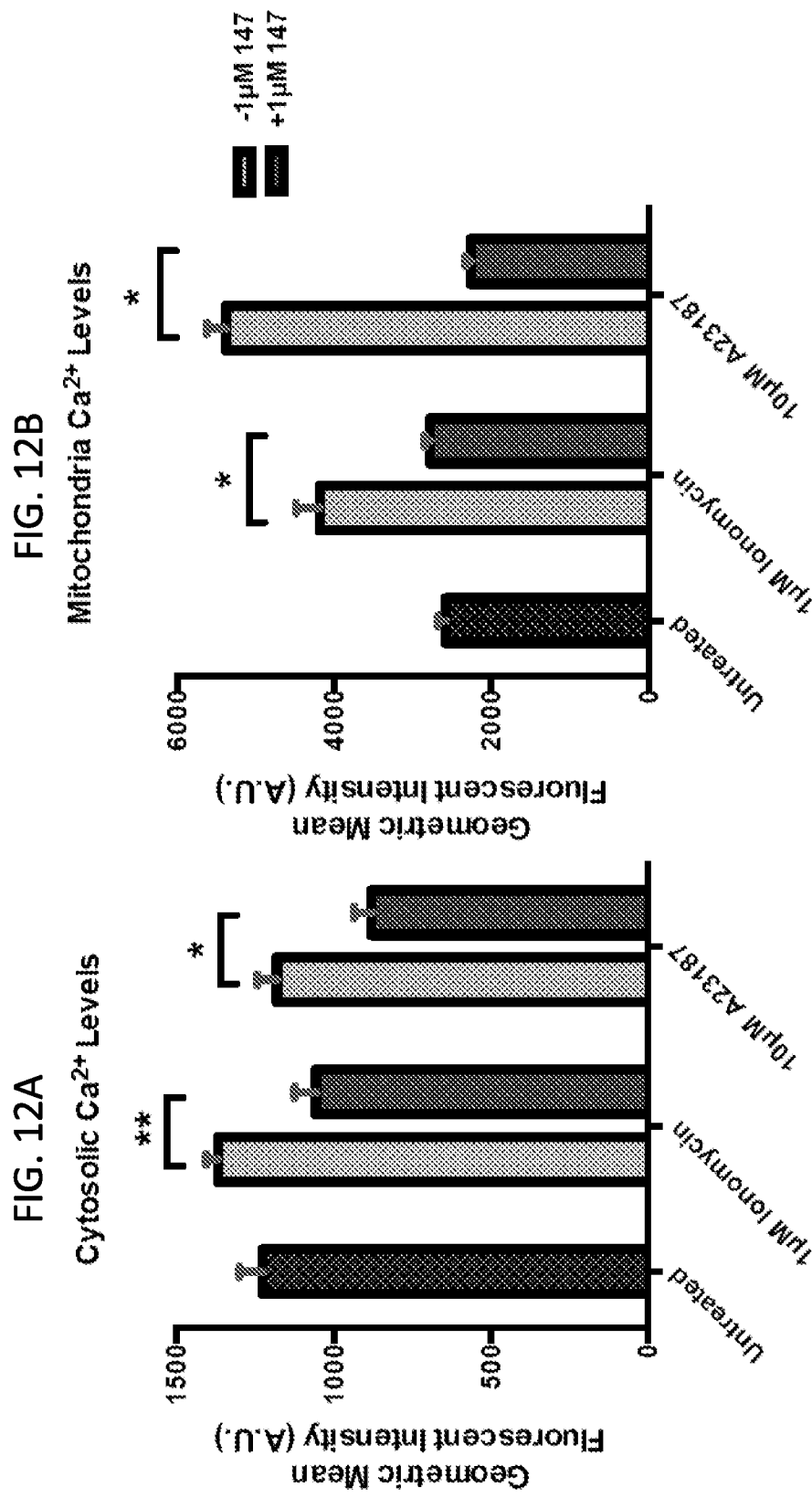


FIG. 13A

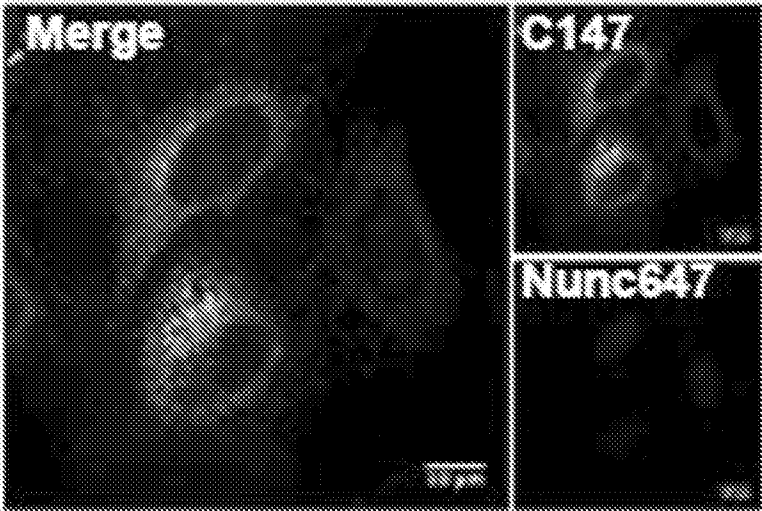


FIG. 13B

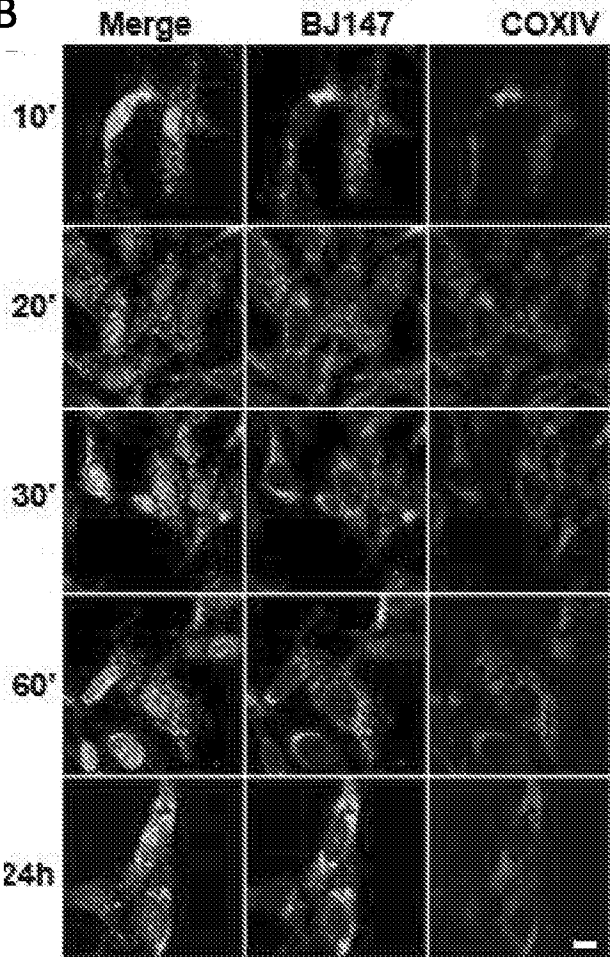


FIG. 13C

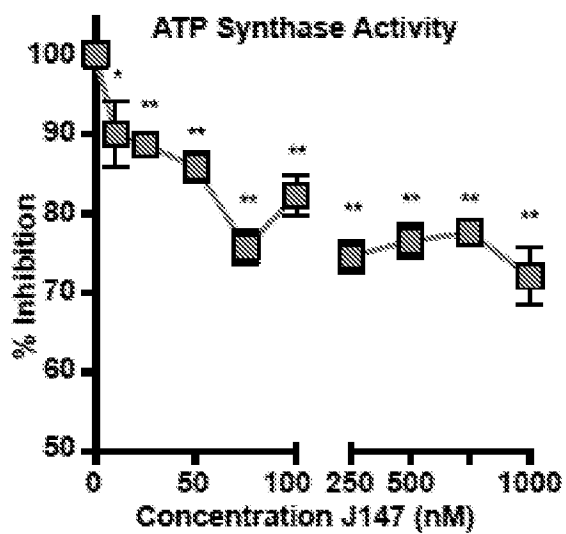


FIG. 13D

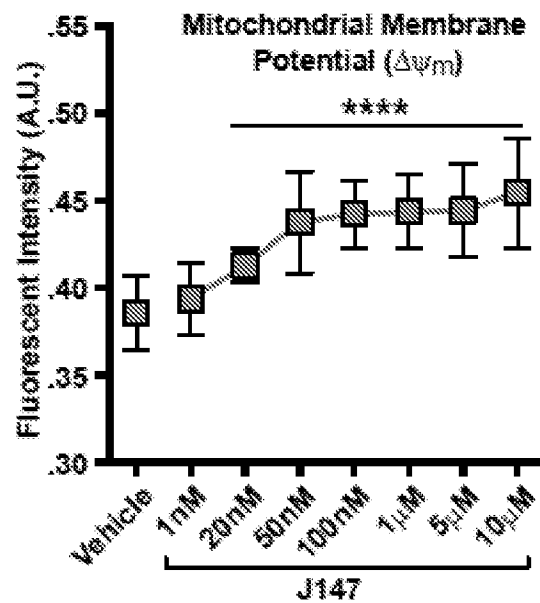


FIG. 13E

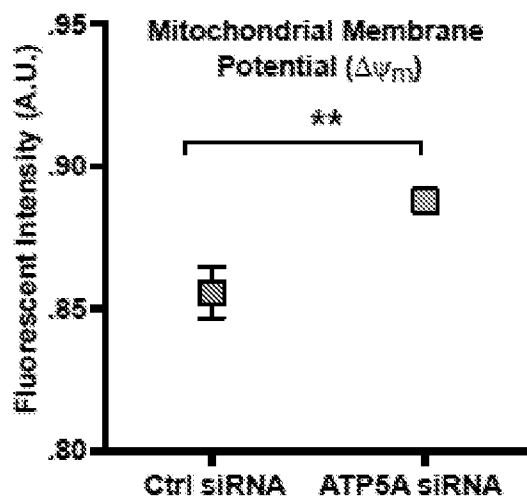
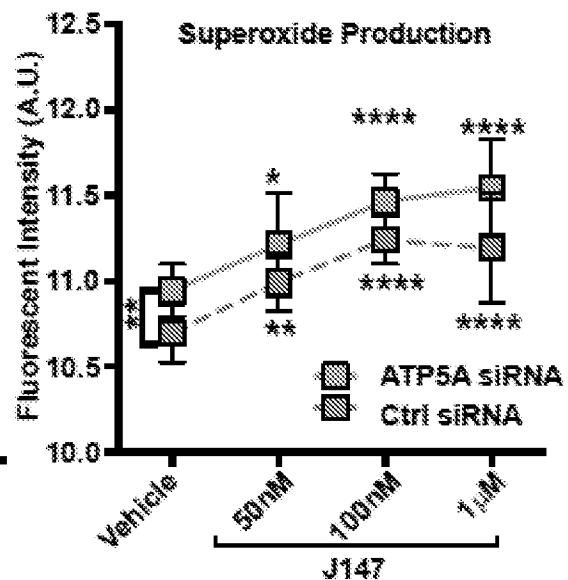


FIG. 13F



J147
 ATP5A siRNA
 Ctrl siRNA or Ctrl vehicle

FIG. 13G

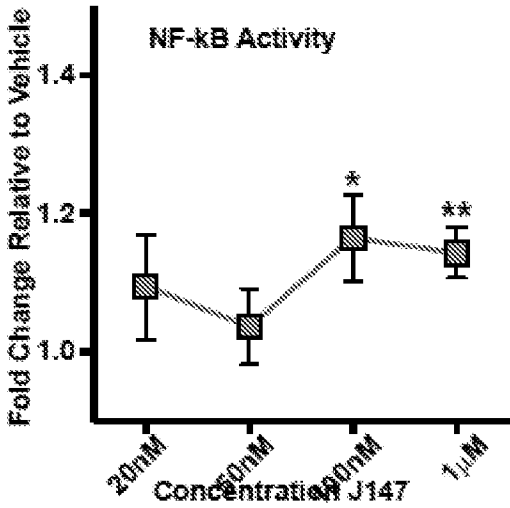


FIG. 13H

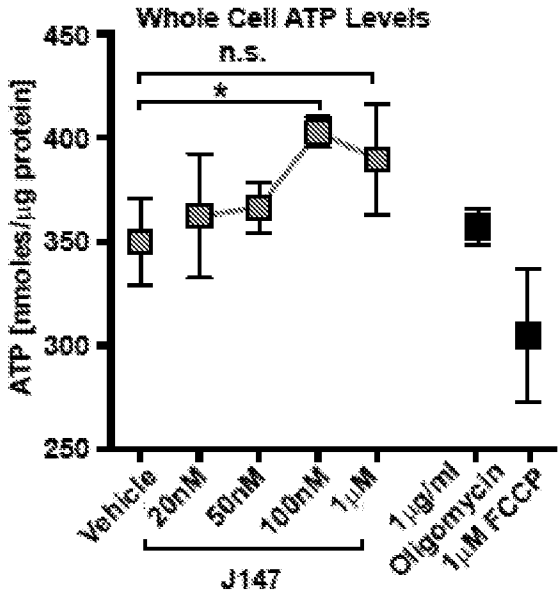


FIG. 13I

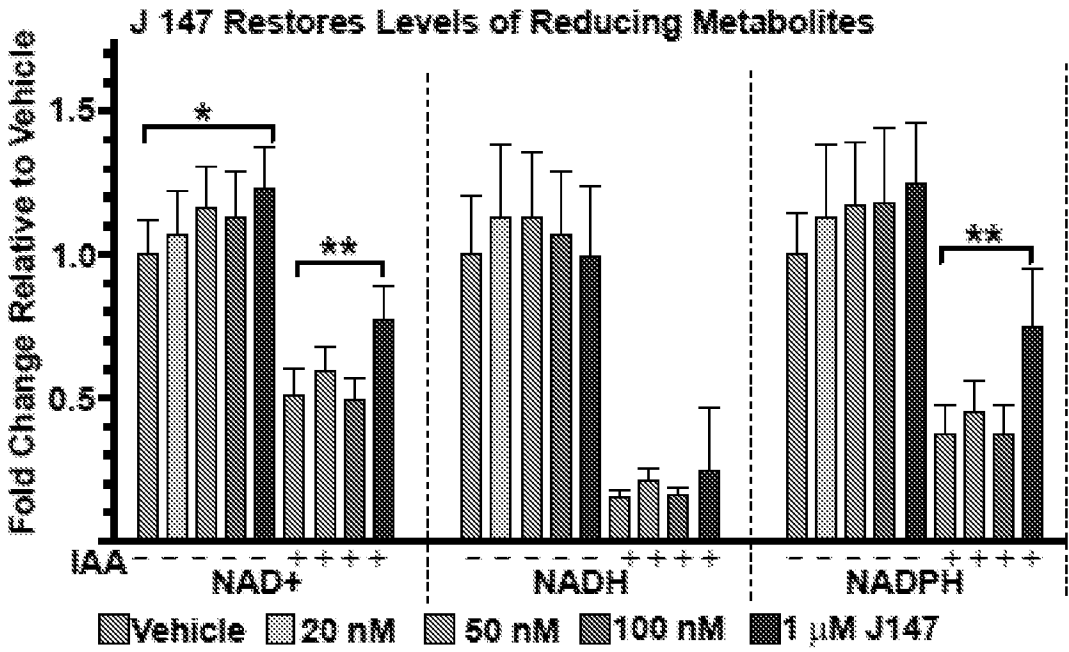


FIG. 14A

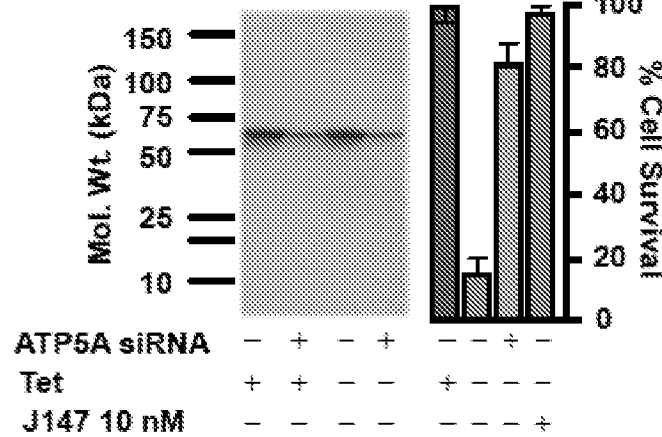
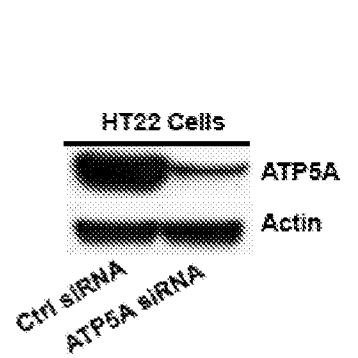


FIG. 14B



■ Ctrl siRNA or Ctrl vehicle ■ ATP5A siRNA
■ 50 nM J147 ■ 100 nM J147 ■ 1 μ M J147

FIG. 14C

ATP Synthase Knockdown Prevents
IAA-Induced Cell Death

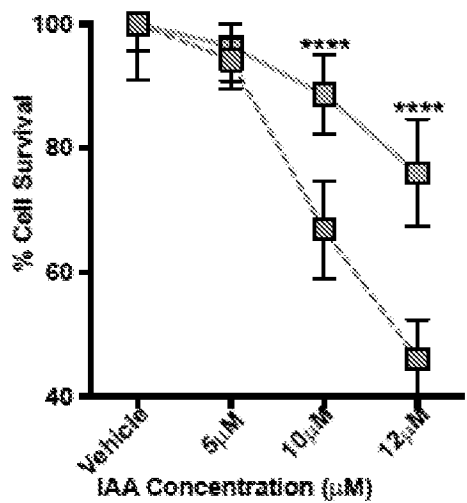


FIG. 14D

Knockdown of ATP Synthase Prevents
Oxytosis-Induced Neuronal Cell Death

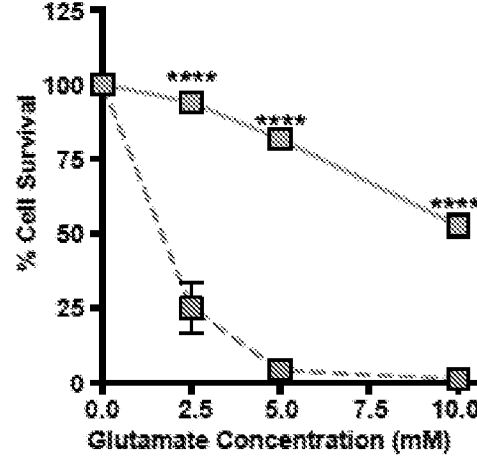


FIG. 14E

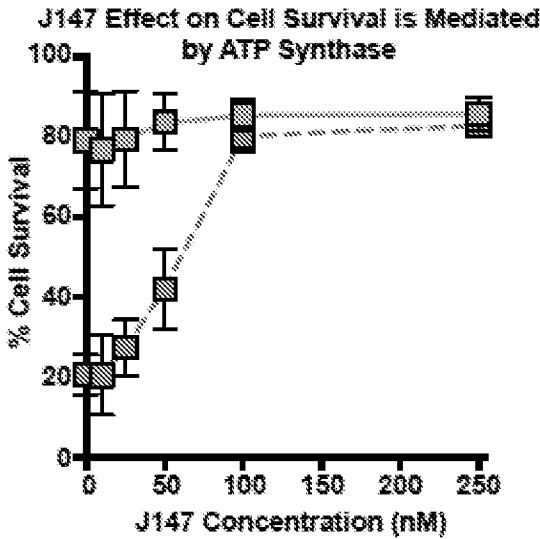


FIG. 14F

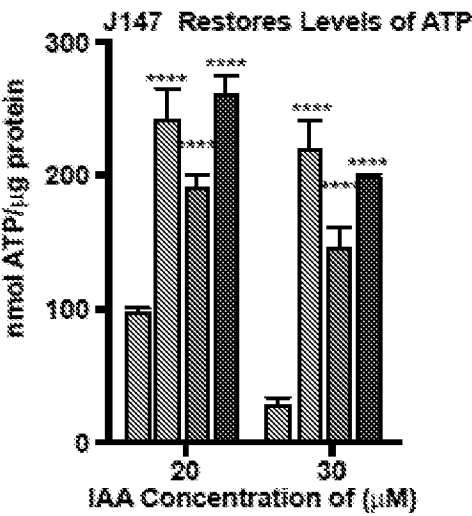


FIG. 14G

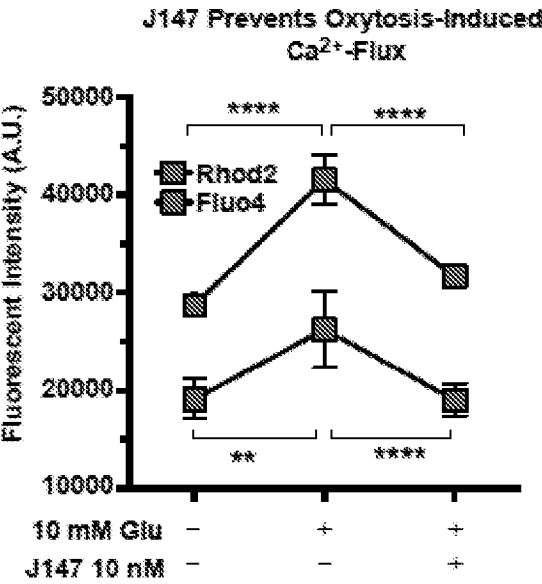


FIG. 14H

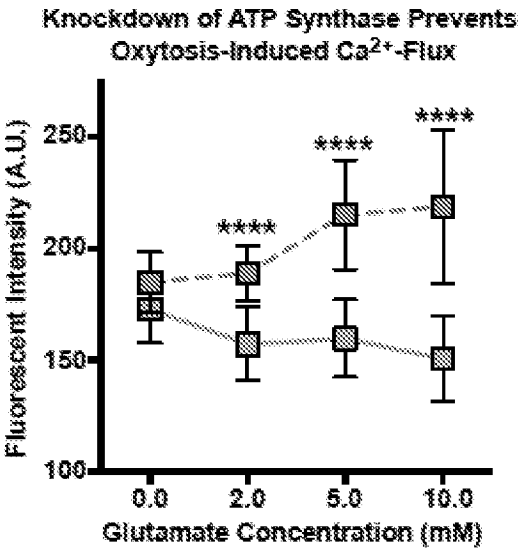


FIG. 15C Cortical Neurons

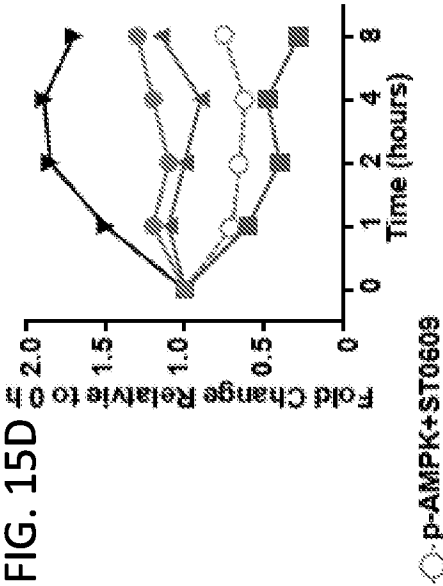
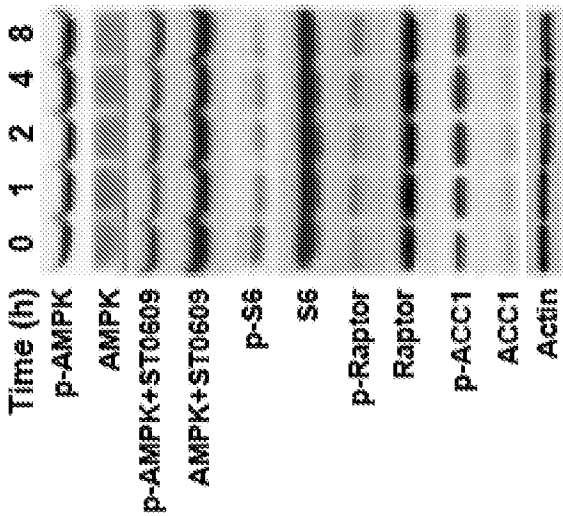


FIG. 15A HT22 MC65

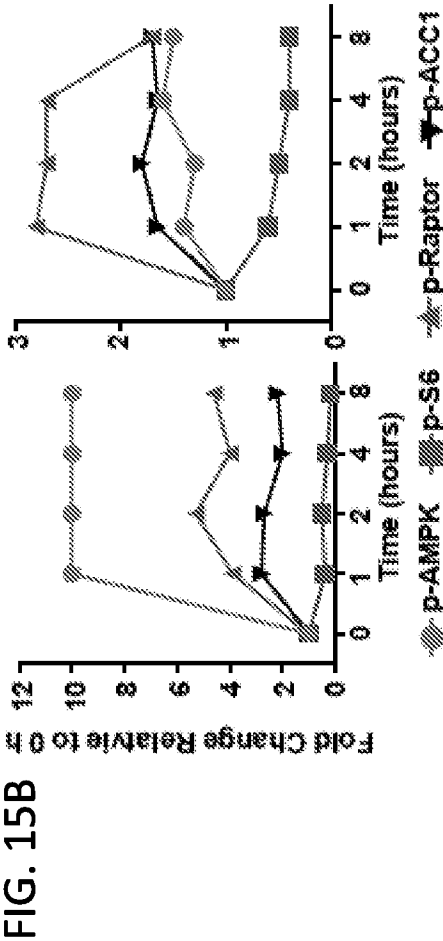
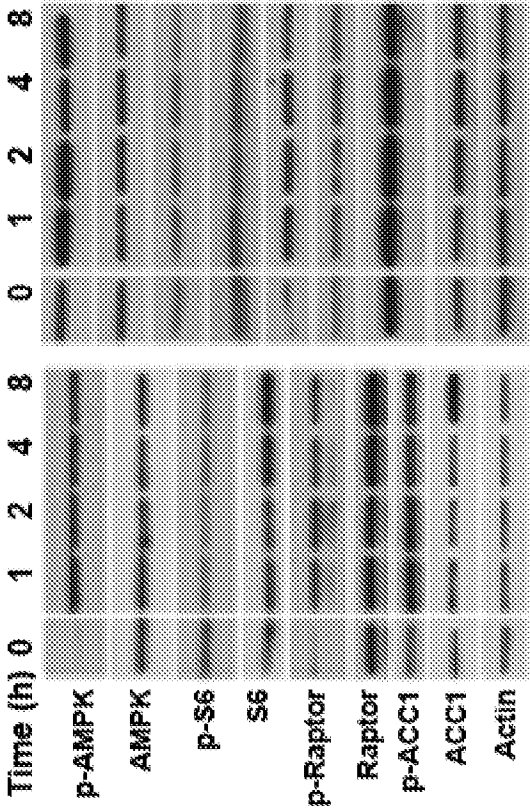


FIG. 16A

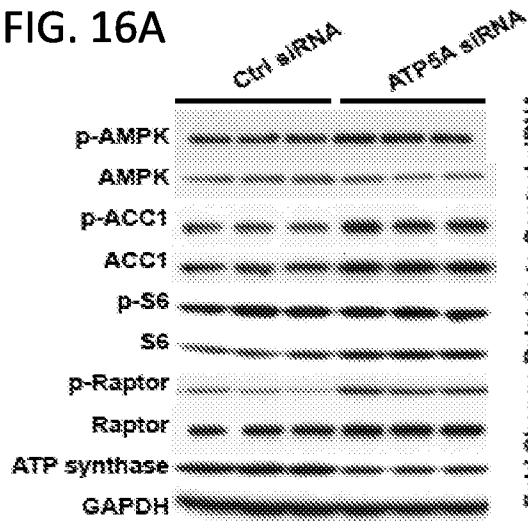


FIG. 16B

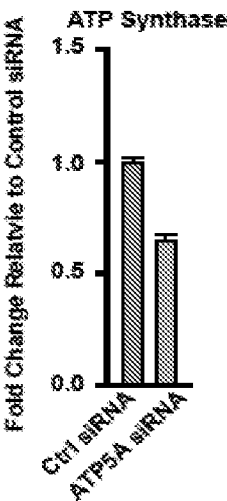


FIG. 16C

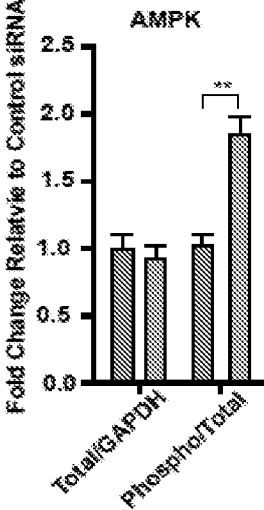


FIG. 16D

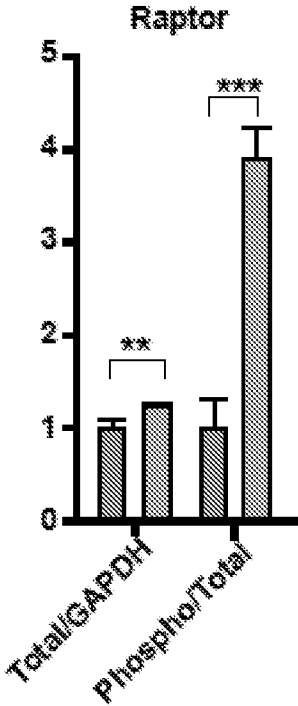


FIG. 16E

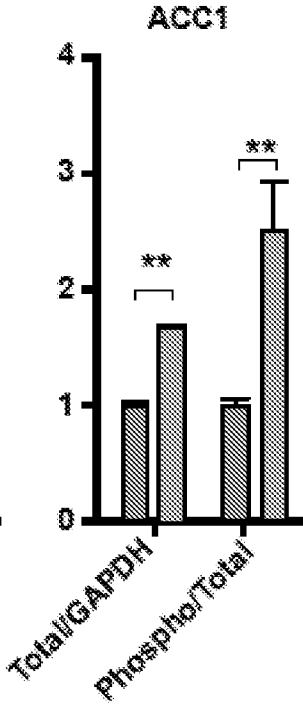


FIG. 16F

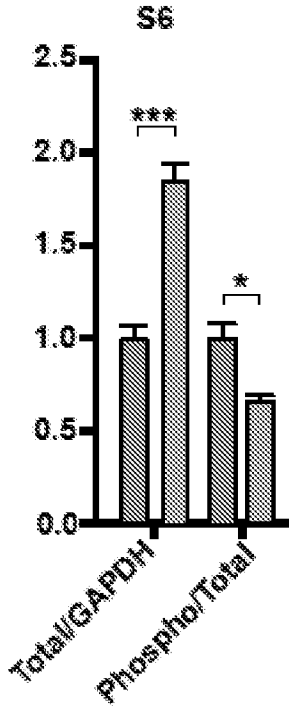


FIG. 17A

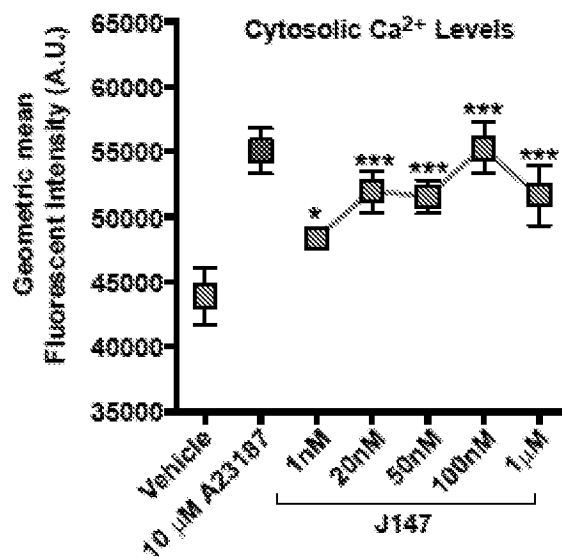


FIG. 17B

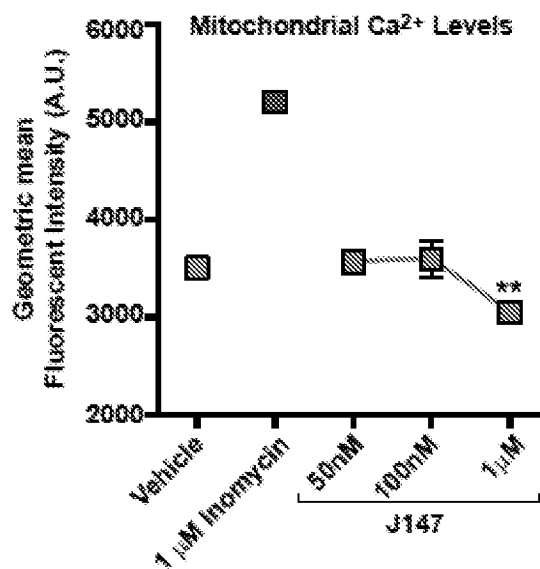


FIG. 18A

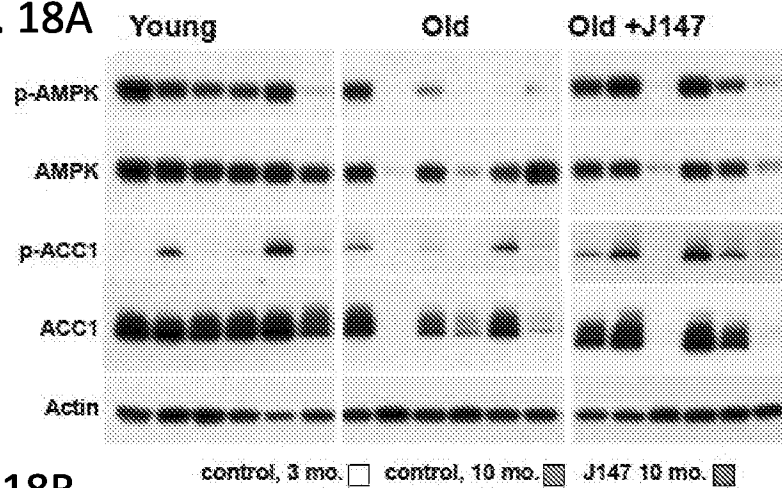


FIG. 18B

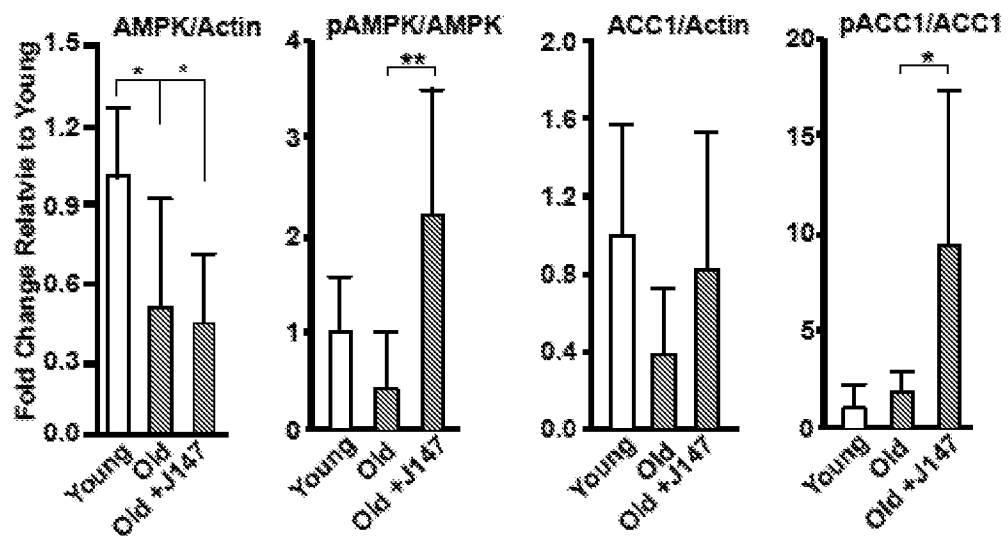


FIG. 18C

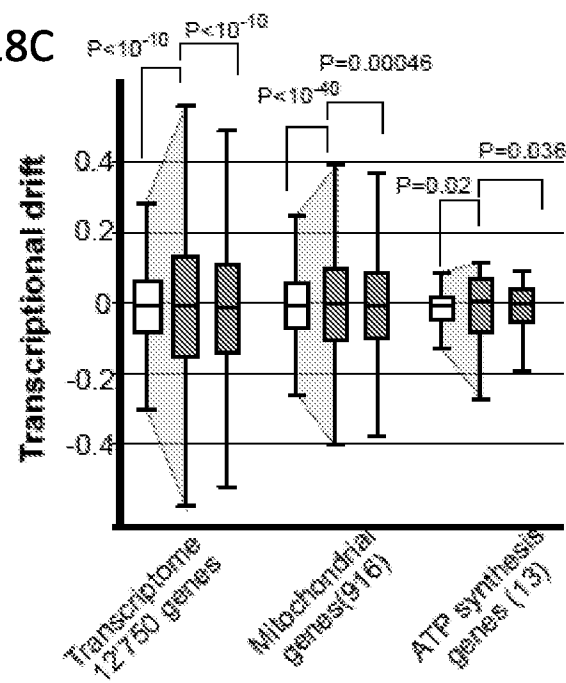


FIG. 18D

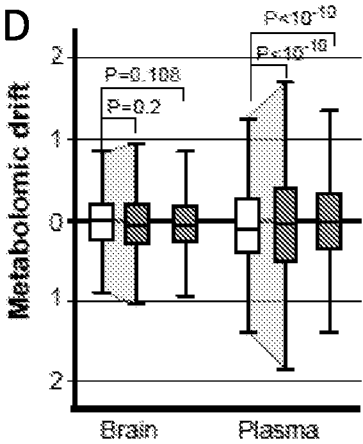


FIG. 18E

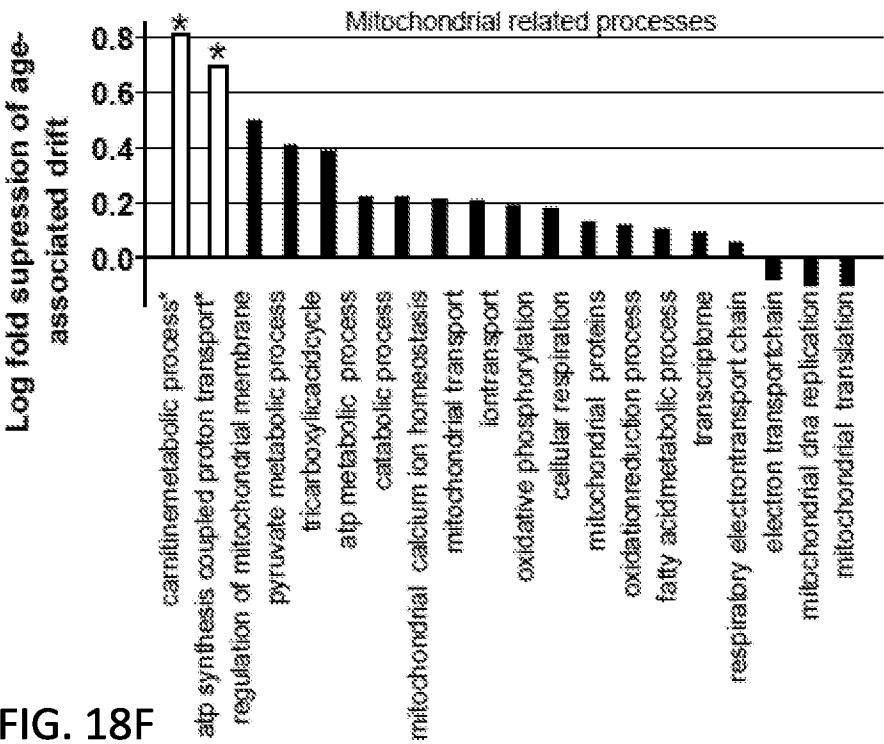


FIG. 18F

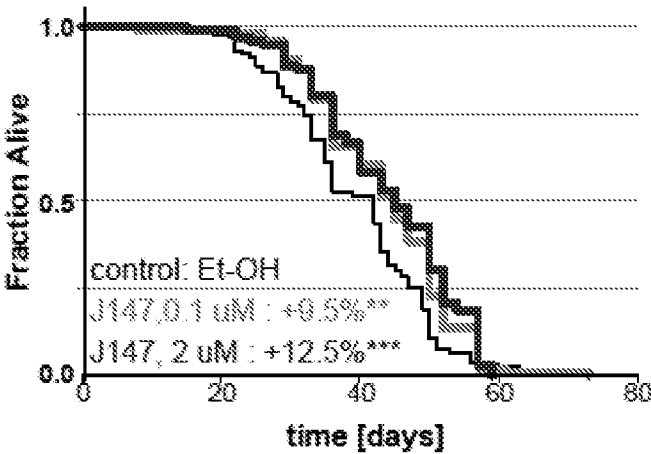


FIG. 19

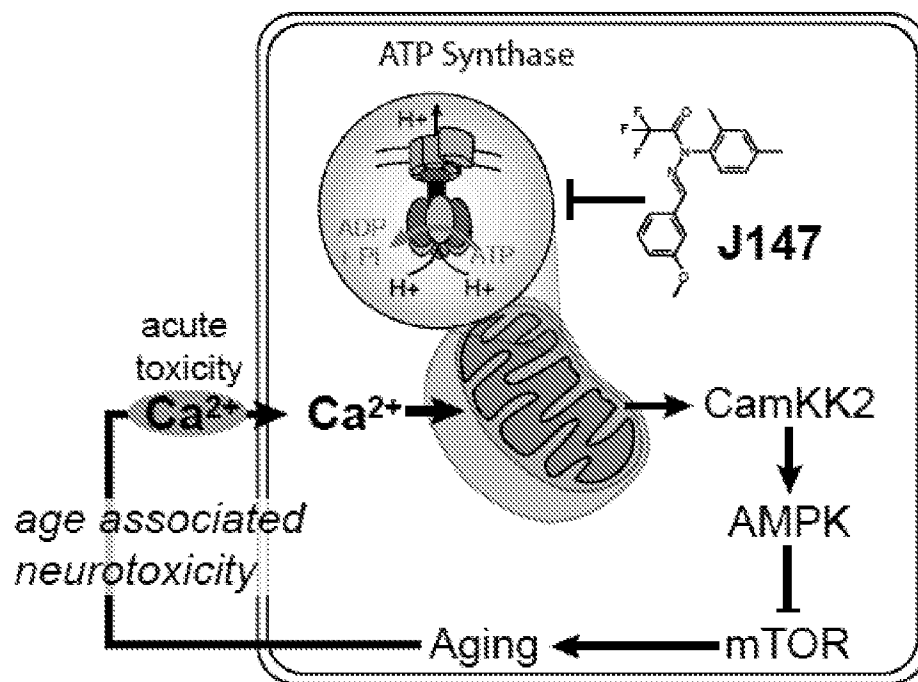


FIG. 20A

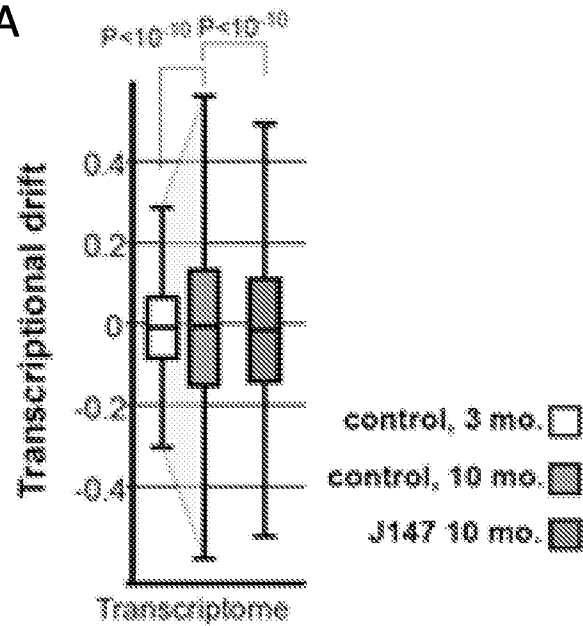


FIG. 20B

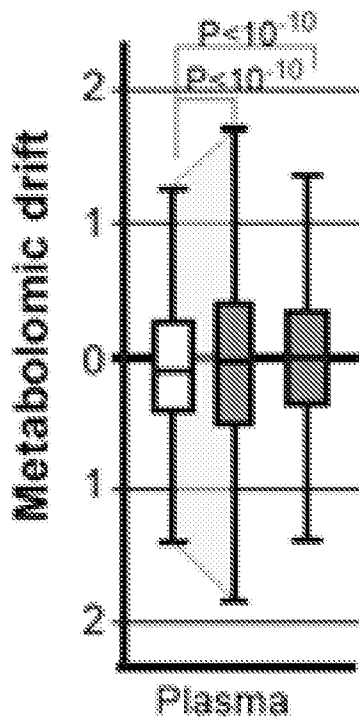


FIG. 20C

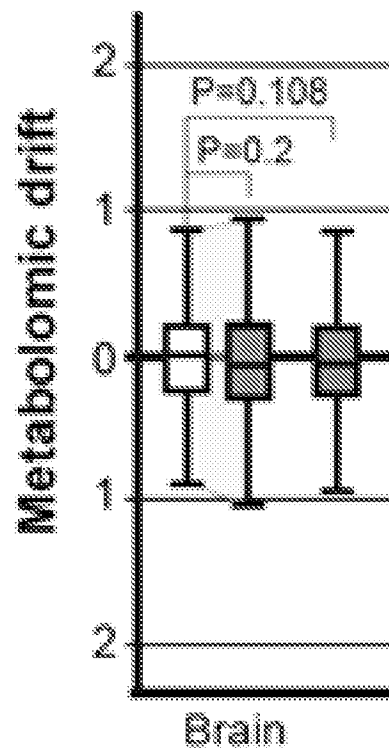


FIG. 21

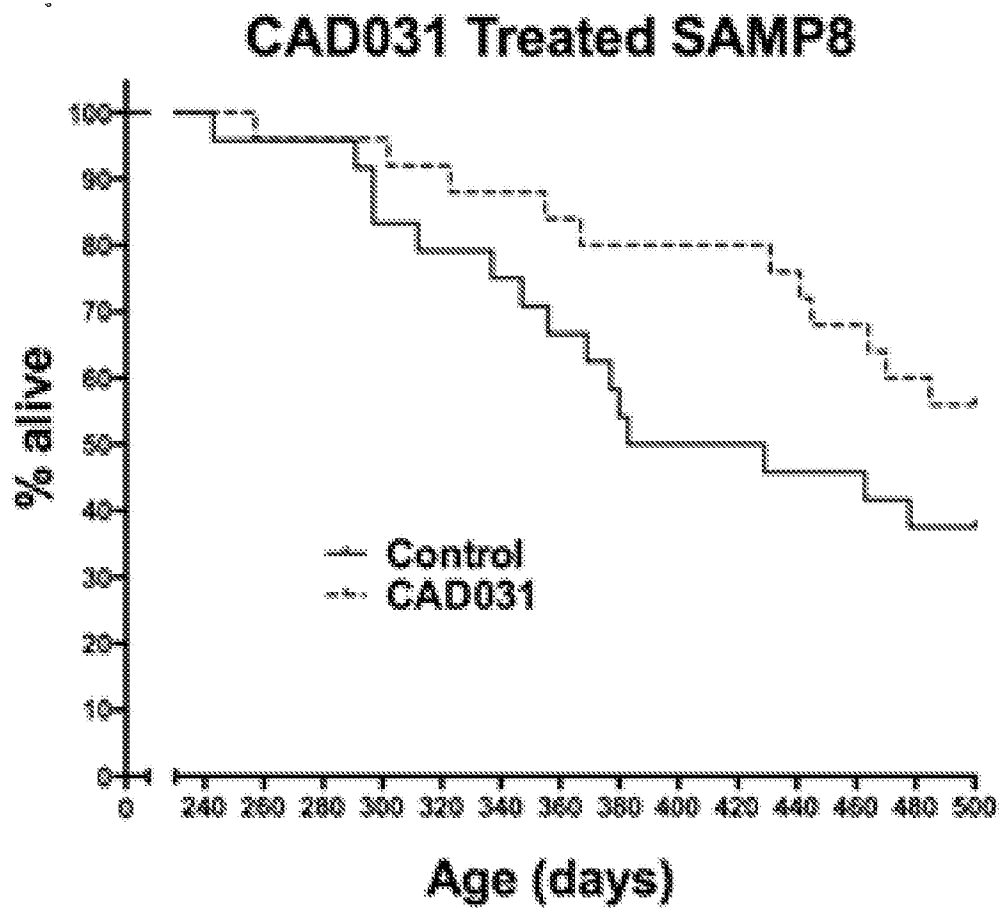


FIG. 22A

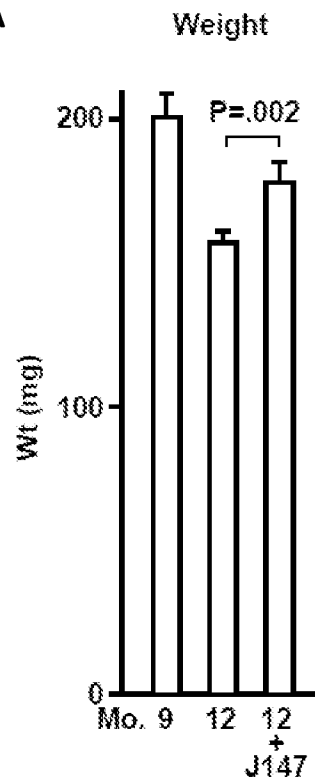


FIG. 22B

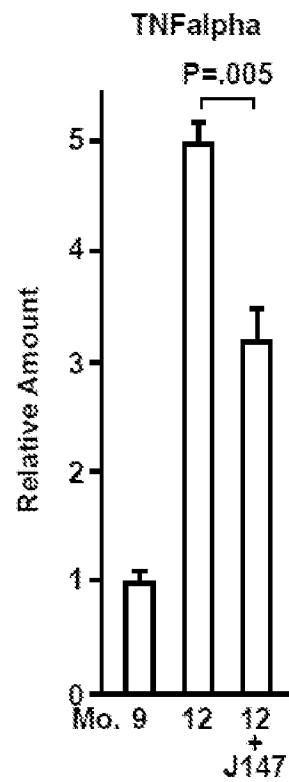


FIG. 22C

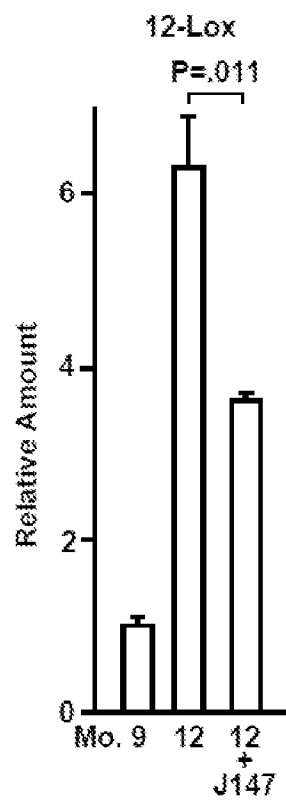


FIG. 22D

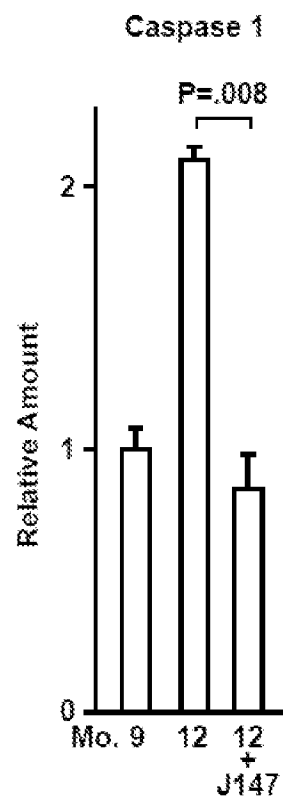


FIG. 23A

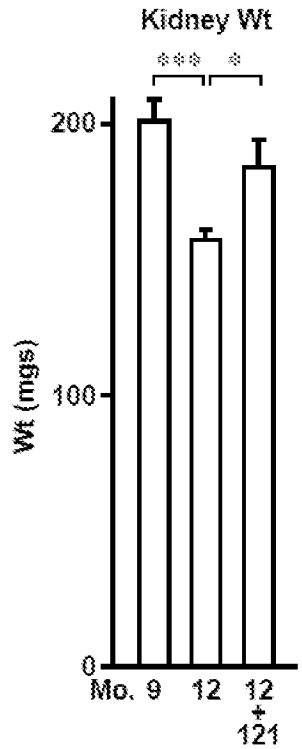


FIG. 23B

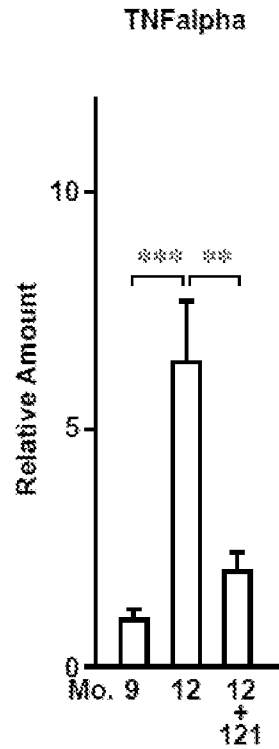


FIG. 23C

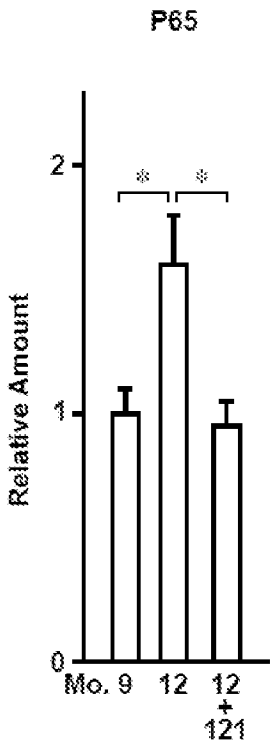
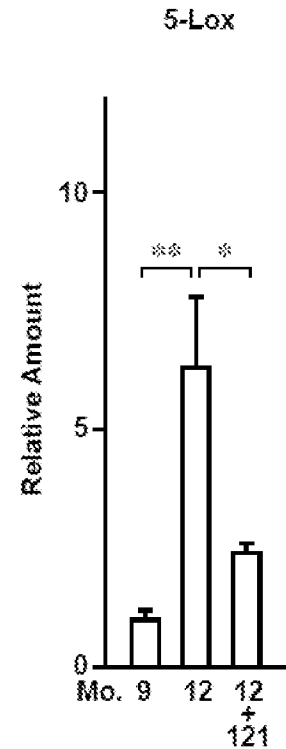


FIG. 23D

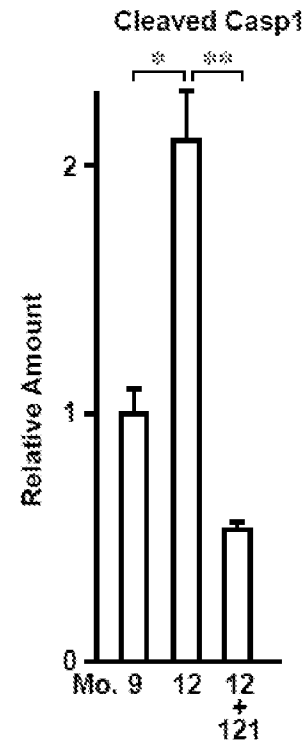


FIG. 23E

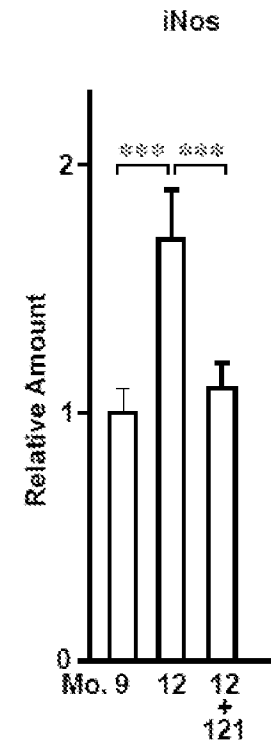


FIG. 23F

INTERNATIONAL SEARCH REPORT

 International application No.
PCT/US2016/043863

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/00 (2006.01) A61K 31/00 (2006.01) G01N 33/50 (2006.01) G01N 33/68 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIAP, EPODOC, CAPLUS, Medline, BIOSIS, EMBASE. Keywords: J147, neurodegenerative disease, mitochondrial permeability transition pore, treatment, prevention, screen, assay and like terms

GOOGLE Scholar was searched with keywords: J147, neurodegenerative disease, mitochondrial permeability transition pore and like terms

Inventor name searches were conducted in Patentscope, PubMed and in internal databases provided by IP Australia

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 28 November 2016		Date of mailing of the international search report 28 November 2016	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au		Authorised officer Alan Brownlee AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61 262832943	

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/US2016/043863
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RAO, V.K. et al., "Mitochondrial permeability transition pore is a potential drug target for neurodegeneration", Biochim. Biophys. Acta (2014), vol. 1842, pages 1267-1272 Abstract; Fig. 2; page 1271, Conclusion, last sentence; page 1268, col. 2, lines 19-25; ref. 64; page 1269, section 2.3	1-15, 20
X	STAVROVSKAYA, I.G. et al., "Clinically approved heterocyclics act on a mitochondrial target and reduce stroke-induced pathology", J. Exp. Med. (2004), vol. 200, pages 211-222 Abstract; Fig. 2; page 212, col. 2, paras 2, 4 and 5; Table I; page 219, col. 1, para. 2 and col. 1, last para - col. 2, first para	1-15, 20
X	MOROTA, S. et al., "Evaluation of putative inhibitors of mitochondrial permeability transition for brain disorders - Specificity vs. toxicity", Exp. Neurol. (2009), vol. 218, pages 353-362 Abstract; Materials and Methods; Table I; Fig. 1; page 354, col. 1, paras 2-4	1-15, 20
X	WO 2004/070054 A2 (CORNELL RESEARCH FOUNDATION, INC.) 19 August 2004 Abstract; [0074], [0080], [0086], Examples 6-8, Figs 2, 3 and claims 1, 47-50	1-5, 8, 11-15, 20
X	WO 2001/032876 A2 (MITOKOR) 10 May 2001 page 5, lines 5-14; page 8, lines 22-24; page 11, lines 5-15; page 12, line 24 - page 13, line 1; page 17, lines 1-25; page 47, lines 13-22; page 60, lines 5-10; Examples 13, 19; page 61, line 28 - page 62, line 3; page 64, lines 22-30; page 65, lines 1-8; claims 37-42, 64-66	1-5, 8, 11-15, 20
X	PRIOR, M. et al., "The neurotrophic compound J147 reverses cognitive impairment in aged Alzheimer's disease mice", Alzheimers Res. Ther. (2013) vol. 5:25 Abstract; Figs 1, 3	11, 13-15, 20
X	CHEN, Q. et al., "A novel neurotrophic drug for cognitive enhancement and Alzheimer's disease", PLoS ONE (2011), vol. 6: e27865 whole document especially Figs. 2-5	11, 13-15, 20
P,X	WO 2016/073633 A1 (UNIVERSITY OF KANSAS et al.) 12 May 2016 [0015], [0262] ff., [0293]-[0297], [0298]-[0302], [0303] ff., [0341] and claims 18-22	1-15, 20

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including
2. ☒ Claims Nos.: **16-19 and 21-27(in full) and 20 (in part)**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
See Supplemental Box
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Supplemental Box**Continuation of Box II**

The claims do not comply with Rule 6.2(a) because they rely on references to the description and/or drawings.

INTERNATIONAL SEARCH REPORT		International application No.			
Information on patent family members		PCT/US2016/043863			
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.					
Patent Document/s Cited in Search Report		Patent Family Member/s			
Publication Number	Publication Date	Publication Number	Publication Date		
WO 2004/070054 A2	19 August 2004	WO 2004070054 A2	19 Aug 2004		
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		Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.			
		Form PCT/ISA/210 (Family Annex)(July 2009)			

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/US2016/043863	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
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End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.			