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Hydroxyurea attenuates oxidative, metabolic, and excitotoxic stress in rat hippocampal neurons and improves spatial memory in a mouse model of Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by accumulation of amyloid β -peptide (A β) plaques in the brain and decreased cognitive function leading to dementia. We tested if hydroxyurea (HU), a ribonucleotide reductase inhibitor known to activate adaptive cellular stress responses and ameliorate abnormalities associated with several genetic disorders, could protect rat hippocampal neurons against oxidative-, excitatory-, mitochondrial-, and A β -induced stress and if HU treatment could improve learning and memory in the APP/PS1 mouse model of AD. HU treatment attenuated the loss of cell viability induced by treatment of hippocampal neurons with hydrogen peroxide, glutamate, rotenone, and A β _{1–42}. HU treatment attenuated reductions of mitochondrial reserve capacity, maximal respiration, and cellular ATP content induced by hydrogen peroxide treatment. *In vivo*, treatment of APP/PS1 mice with HU (45 mg/kg/day) improved spatial memory performance in the hippocampus-dependent Morris water maze task without reducing A β levels. HU provides neuroprotection against toxic insults including A β , improves mitochondrial bioenergetics, and improves spatial memory in an AD mouse model. HU may offer a new therapeutic approach to delay cognitive decline in AD.

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Keywords

hydroxyurea; amyloid beta; Alzheimer's disease; APP/PS1; neurodegeneration; mitochondria

1. Introduction

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder that affects more than 5 million Americans age 65 or older and is expected to affect nearly 14 million Americans by 2050 (Hebert et al., 2013). Individuals with AD experience episodic memory loss and dementia (Association, 2016). AD histology is characterized by the accumulation of amyloid β -peptide ($A\beta$) plaques and neurofibrillary tangles composed of hyperphosphorylated tau protein (Polanco et al., 2018). Brains from individuals with AD also exhibit an increase in inflammation characterized by activated astrocytes and microglial cells (Van Eldik et al., 2016). At the cellular level, neurons affected in AD exhibit mitochondrial dysfunction, impaired autophagy, increased oxidative stress, and an impaired ability to respond to and repair oxidative damage (Stranahan and Mattson, 2012).

Treatments are not available to prevent or slow the progression of AD. Currently, individuals with moderate to severe AD may be prescribed drugs such as memantine, an NMDA receptor antagonist, or acetylcholinesterase inhibitors, that have small effects on clinical symptoms (Aisen et al., 2012). However, there is currently no treatment available that substantially delays the onset or progression of AD. Due to a lack of prevention and an increase in prevalence, the current estimated cost of AD and other dementias in the United States alone is over 250 billion dollars annually. It is anticipated to rise to \$1.1 trillion dollars by 2050 if an effective intervention(s) is not discovered (Association, 2016).

Previous studies in AD animal models have provided evidence that physiological and pharmacological interventions that activate adaptive cellular response signaling pathways, such as intermittent fasting and caloric restriction, can protect neurons against dysfunction and degeneration. For example, daily caloric restriction reduces $A\beta$ pathology in amyloid precursor protein (APP) mutant mice and in APP^{swe695}/PS1^{delE9} (APP/PS1) double mutant transgenic mice (Patel et al., 2005). Intermittent fasting (alternate day fasting) and 30% daily caloric restriction ameliorate cognitive decline during aging in 3xTg AD mice (Halagappa et al., 2007). Intermittent fasting induces components of the adaptive cellular stress response and leads to increased neuronal resistance to stress as well as improved synaptic plasticity (Mattson et al., 2018). Treatment of APP mutant mice with the mTOR inhibitor rapamycin stimulated neuronal autophagy, reduced $A\beta$ pathology and lessened cognitive deficits (Spilman et al., 2010). Treatment of 3xTg AD mice with 2-deoxyglucose, which induces mild metabolic stress, bolstered mitochondrial function and reduced $A\beta$ neuropathology (Yao et al., 2011). We previously found that hydroxyurea (HU), a ribonucleotide reductase inhibitor, improves cellular metabolic defects in fibroblasts from several genetic disorders and activates adaptive cellular stress responses in cultured human fibroblasts (Brose et al., 2012). These include up-regulation of antioxidant defenses, protein chaperones and autophagy, and stimulation of mitochondrial biogenesis. Here we show that

HU treatment protects cultured hippocampal neurons against oxidative, metabolic and excitotoxic stress, and improves memory in the APP/PS1 mouse model of AD.

2. Methods

2.1 Rat hippocampal neuronal cultures

Primary hippocampal neuron cultures were prepared from embryonic day 18 Sprague-Dawley rat brains as described previously (Kaech and Banker, 2006; Mattson et al., 1989). Dissociated neurons were plated at a density of 40,000 neurons per well (96-well plate) or 1×10^6 cells per well (6-well plate) on polyethyleneimine-coated plastic dishes in MEM medium supplemented with 10% fetal bovine serum. Once the neurons attached to the plates, the cell plating medium was replaced with Neurobasal (NB) medium with 5% B27 supplement, 1% glutamax, and 1% antibiotic-antimycotic (Invitrogen, Waltham, MA) for 6–7 days. Prior to the addition of HU (5 μ M unless otherwise specified; H8627, Sigma-Aldrich, St. Louis, MO), the neurons were washed with unsupplemented NB medium to remove traces of antioxidants in the B27 supplement, and the medium was replaced with NB medium supplemented with 5% B27 minus antioxidants (NB + B27 – AOX; Invitrogen), 1% glutamax, and 1% antibiotic-antimycotic. Experiments were performed between days *in vitro* (DIV) 6 and 9.

2.2 Neurotoxicity assays

On DIV 6 or 7, neurons were treated with HU (5 μ M; 2.5 μ M for A β _{1–42} experiment). The concentration of HU was titrated to allow 100% cell viability. After 24 hours, H₂O₂ (15 μ M), glutamate (50 μ M; Sigma-Aldrich), rotenone (75 nM; Sigma-Aldrich), or oligomerized A β _{1–42} peptide (5–10 μ M; Bachem, Torrance, CA) was added and the neurons cultured for another 24 hours. For the A β _{1–42} peptide experiments, the neurons were incubated with HU (2.5 μ M) in NB + B27 – AOX for the first 24 hours. The medium was then changed to NB medium without any B27 supplement plus HU (2.5 μ M); and, A β _{1–42} peptide added for the last 24 hours of incubation. Without B27 supplements the neurons are more sensitive to perturbations. Therefore, the HU dosage was titrated to 2.5 μ M to allow 100% cell viability. Cell viability was assessed using an MTS cell viability assay per manufacturer's instructions (CellTiter Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI). Briefly, 20 μ l of CellTiter Aqueous One Solution was added to cells in 100 μ l of NB medium + B27 – AOX. Cells were incubated at 37° C in 5% CO₂, and the A₄₉₀ measured with a Synergy H1 Microplate Reader (Biotek, Winooski, VT). Since metabolically active cells reduce the MTS tetrazolium compound to a colored formazan product, the amount of formazan measured at A₄₉₀ was proportional to the number of metabolically active cells. Cell viability was normalized to the untreated control cells (100% viability).

2.3 Mitochondrial activity measurements

Mitochondrial activity was assessed using a Seahorse XF-96 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA) according to the manufacturer's instructions and as described previously (Divakaruni et al., 2014; Yao et al., 2017). Briefly, neurons were grown in Seahorse 96-well plates (40,000 cells/well). On DIV 6, the media was changed to NB + B27 minus AOX. Neurons were treated with HU (5 μ M) on DIV 7. After 24 hours, the

neurons were treated with H₂O₂ (35 μ M) for one hour. The medium was changed to unbuffered XF assay medium (Seahorse Biosciences) supplemented with 5 mM glucose, 1 mM pyruvate, and 2 mM glutamax (Invitrogen). The neurons were incubated in a non-CO₂ incubator at 37°C for one hour followed by measurement of basal oxygen consumption rate (OCR) and OCR after the sequential addition of 2 μ M oligomycin, 1 μ M FCCP and 5 μ M rotenone/antimycin A. Oligomycin inhibits mitochondrial ATP synthase activity and facilitated the measurement of ATP-linked respiration. FCCP uncouples the mitochondrial proton gradient and disrupts the mitochondrial membrane potential allowing measurement of maximal respiration. Rotenone inhibits mitochondrial complex I and antimycin inhibits mitochondrial complex III. The use of rotenone and antimycin together shuts down mitochondrial respiration and allows measurement of non-mitochondrial respiration.

2.4 ATP content

Cellular adenosine triphosphate (ATP) content was determined using the ATP Determination Kit (#A22066; Molecular Probes, Eugene, OR) following the manufacturer's instructions. Briefly, on DIV 7, hippocampal neurons were treated with HU (5 μ M). Approximately 24 hours later, H₂O₂ peroxide was added (15 μ M or 35 μ M) for one hour. All medium was removed, 11 μ l boiling water was added to each well, and 110 μ l of ATP reaction mixture was added and mixed with the lysed cells. ATP content was determined by measuring luciferase activity compared to a standard curve. The concentration of ATP per treatment condition was normalized relative to the untreated control neurons. N = 3 independent experiments.

2.5 RNA isolation and microarray analyses

RNA was isolated from rat hippocampal neurons treated with HU (5 μ M) for 2, 6, 12, or 24 hours (n = 3 per time point) and from frozen mouse hippocampi isolated from 19 to 24-week-old nontransgenic control littermates of APP/PS1 mice treated with HU (45 mg/kg/day; n = 4) or vehicle control (water; n = 4) for 2.5 months using Trizol and following the manufacturer's instructions (Thermo Fisher Scientific). Animal housing conditions are listed below in section 2.8. Total RNA was quality-controlled on the Agilent Bioanalyzer RNA 6000 Chip (Agilent, Santa Clara, CA), and 200 ng labeled using the Agilent Low-Input QuickAmp Labeling Kit, and purified and quantified according to the manufacturer's instructions. A total of 600 ng Cy3labeled cRNA was hybridized for 17 hours to Agilent SurePrint G3 Rat and Mouse GE 8 $\times$ 60K microarrays. Following post-hybridization rinses, arrays were scanned using an Agilent SureScan microarray Scanner, and hybridization intensity data extracted from the scanned images using Agilent's Feature Extraction Software. Raw data were analyzed by Z-normalization as described previously (Cheadle et al., 2003). Principal component analysis (PCA) was performed on the normalized Z-scores of all detectable probes using DIANE 6.0 software (http://www.grc.nia.nih.gov/branches/rrb/dna/diane_software.pdf). For the hippocampal samples, PCA analysis identified the data of one untreated wild type hippocampus and one HU-treated wild type hippocampus as outliers. Significance of gene expression differences between control and HU-treated neurons or hippocampi were based on a Z-test < 0.05, false discovery rate < 0.30, and a Z-ratio > 1.5 in either direction, and ANOVA p value < 0.05. The raw and normalized data sets are deposited in NCBI's Gene Expression Omnibus (GEO) and are

accessible through GEO Series accession number GSE111943 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE111943>), with the individual data sets available as GSE111941 (rat neurons) and GSE111940 (mouse hippocampi).

2.6 Western blot analyses

Neuronal cell lysates were collected in RIPA buffer [50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.2 mM sodium orthovanadate, 1 mM PMSF, plus 1 x protease inhibitors (#11 836 153 001, Roche, Indianapolis, IN)] and stored at -80°C . Denatured LDS-PAGE and immunoblot analyses were performed using the following antibodies: total OXPHOS rodent WB antibody cocktail (ab110413, Abcam, Cambridge, MA), LC3 (NB100-2220, Novus Biologicals, Littleton, CO), β -actin (A5441, Sigma-Aldrich, St. Louis, MO), and SIRT3 (#5490, Cell Signaling, Danvers, MA). Blot imaging and protein quantification were performed using an Odyssey Imager (Li-Cor Biosciences, Lincoln, NE). β -actin levels were used for normalization.

2.7 Proteasome activity assay

20S and 26S proteasome activity levels were measured using a Proteasome Activity Assay Kit (#APT280; Millipore, Burlington, MA) following the manufacturer's directions. The following modifications were made to measure 20S and 26S proteasome activity. Briefly, HU-treated neurons (6×10^6) were harvested on DIV 8 in 50 μl RIPA and stored at -80°C . The lysate was suspended in assay buffer supplemented with 10 mM MgCl_2 and divided into two samples. Sample one contained 15 mM 2-deoxyglucose and hexokinase (0.1 mg/ μl) to deplete ATP activity and measure 20S proteasome activity. Sample two was supplemented with 2 mM ATP to measure 26S proteasome activity. Proteasome activity was measured by the detection of free fluorophore 7-amino-4-methylcoumarin (AMC; fluorescence 380/460 nm) after cleavage from the proteasome substrate LLVY-AMC.

2.8 Animal behavior testing study design

All studies were approved by the National Institute of Aging Institute of Animal Care and Use Committee and all procedures followed the NIH Guide for the Care and Use of Laboratory Animals. All mice were female APP^{swe695}/PSEN1^{dE9} mice [B6.Cg-Tg (APP^{swe},PSEN1^{dE9}) 85Dbo/J; APP/PS1] or their nontransgenic (NTG) littermate controls. Original breeding pairs were purchased from the Jackson Laboratory, and were maintained on a C57BL/6J background in the National Institute of Aging vivarium under a 12-hour light/12-hour dark cycle. There were two to four mice per cage. HU-treated mice were given 45 mg/kg/day of HU in their drinking water. The dosage was based on the dosage used in sickle cell disease mouse models (50mg/kg/day; (Lebensburger et al., 2012). The dosage in mice is higher than in humans. Individuals with sickle cell disease, psoriasis, or HIV are administered 15 mg/kg/day and not to exceed 35 mg/kg/day (Medscape, 2018). Two cohorts were studied; cohort 2 was set-up after the completion of studies with cohort 1. Cohort 1 consisted of 11 untreated NTG control littermates (29.31g $\pm$ 0.91 SEM), seven untreated APP/PS1 mice (27.44g $\pm$ 0.72 SEM), and eight HU-treated APP/PS1 mice (26.66g $\pm$ 1.16 SEM). One HU-treated APP/PS1 mouse was blind and excluded from behavioral testing. HU treatment began at two to four months of age. Behavioral testing started at six to eight months of age. The total treatment length was five months. Cohort 2 consisted of nine

untreated NTG control mice (28.70 $\pm$ 1.31 SEM), eight HU-treated NTG control mice (29.48 $\pm$ 1.23 SEM), 13 untreated APP/PS1 mice (28.89g $\pm$ 0.48 SEM), and 13 HU-treated APP/PS1 mice (30.36 $\pm$ 0.74 SEM). HU treatment started at two and half to five months of age and behavioral testing began at 10.5 to 13 months of age. The total treatment time was nine months. Prior to each behavioral test, animals were habituated to the testing room for 12 hours, two days in a row. One week after behavioral testing, all animals were euthanized and brain tissues were rapidly frozen using dry ice. Within each cohort, the average weight per genotype and treatment group was similar (One-way ANOVA $p_{\text{cohort 1}} = 0.137$, $p_{\text{cohort 2}} = 0.511$).

2.9 Open field test

Mice were acclimated to the room for 30 minutes prior to the first test. The mice were placed in a clear acrylic arena (40.6 cm x 40.6 cm) housed in a sound-attenuating cubicle (Med Associates, St. Albans, VT) for 30 minutes to assess their locomotor and exploratory behavior. Data were collected using Activity Monitor software (Version 4.0, Med Associates). The total distance, total ambulatory time, distance in the center zone, and time in the center zone were measured.

2.10 Elevated plus maze

The elevated plus maze was used to assess anxiety-related behavior. The acrylic apparatus consisted of two open arms (30 cm x 5 cm) and two closed arms (30 cm x 5 cm x 16 cm) raised 60 cm above the floor. Each animal was placed in the center of the apparatus and the animal movement tracked for five minutes with ANY-maze software (Stoelting Co., Wood Dale, IL). The time spent in the open arms and the time spent in the closed arms plus the middle zone were measured.

2.11 Morris water maze

To assess spatial learning and memory, a circular pool (160 cm) was filled with water (22–25°C) and the water rendered opaque by adding enough non-toxic white tempura paint to hide a rectangular platform (14 cm x 14 cm) in the center of the target quadrant two centimeters below the water surface. Distal spatial cues were placed on the walls of the room. During the training phase, each mouse performed four consecutive 60-second swim trials for the specified number of days. For each trial, the mouse was placed facing the wall in a randomly predetermined quadrant such that the mouse started from all four quadrants throughout the four daily trials. If the mouse found the platform, the trial was stopped and the mouse given 15 seconds to sit on the platform before being removed from the water. If the mouse did not find the platform within the allotted 60 seconds, the mouse was guided visually by placing a finger on top of the platform or by manually guiding the mouse to the platform and allowed to sit on the platform for 15 seconds before being removed from the water. Probe trials were performed 24, 48, and 72 hours following the last training trial. For each probe trial, the platform was absent and each mouse was given one 60 second trial to swim in the pool. Following the 72-hour probe trial, a visual platform test was performed to identify any visual discrepancies in the mice. The test was performed in a separate room with a similar pool except paint was not added to the water, there were no distal cues on the walls, and the platform was identified with two flags. Each mouse was given four non-

consecutive 60 second trials to locate the platform. Each mouse was tested with the platform in one quadrant before moving the platform to a different quadrant and performing the next trial round. All trials were video recorded and analyzed using ANY-maze software (Stoelting Co., Wood Dale, IL).

2.12 A β ₄₂ and A β ₄₀ Immunoassay

A β ₄₂ and A β ₄₀ peptide levels were measured by a V-PLEX immunoassay (4G8; Meso Scale Discovery, Rockville, MD) using 1 μ g of mouse cortical homogenates and 2.5 μ g of mouse hippocampal homogenates.

2.13 Statistical analyses

All data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA). Data are represented as the mean $\pm$ standard error of the mean (SEM). Neuronal experiments were performed independently three or more times. Student t-tests were used to compare before and after treatments. One-way ANOVAs followed by *post-hoc* Bonferroni or *post-hoc* Dunnett's multiple comparison tests were used to analyze data with three or more groups. MWM training parameters were analyzed using a two way repeated measures ANOVA (RM-ANOVA) followed by *post-hoc* Bonferroni analyses.

3. Results

3.1 Hydroxyurea treatment protects hippocampal neurons against neurotoxic stress

We hypothesized that HU treatment can upregulate one or more adaptive cellular stress response pathways and thereby protect hippocampal neurons against insults relevant to the pathogenesis of various neurodegenerative disorders. To test this, we investigated the ability of HU to protect wild type rat hippocampal neurons against four neurotoxic agents. The neurotoxic agents investigated included hydrogen peroxide to mimic increased cellular oxidative stress, glutamate to induce excitotoxicity, rotenone to impair mitochondrial function by inhibiting complex I of the mitochondrial electron transport chain, and A β ₁₋₄₂ to mimic the A β aggregation and accumulation that occurs in the brains of AD patients (Camandola and Mattson, 2017; Dumont and Beal, 2011). We examined these effects in wild type rat hippocampal neurons rather than mouse APP/PS1 hippocampal neurons to allow applicability of these results to various neurodegenerative disease models.

Rat hippocampal neurons were pretreated with HU for 24 hours, then co-treated with HU and a neurotoxin for an additional 24 hours. Each neurotoxin significantly reduced cell viability compared to untreated neurons ($p < 0.05$; Table 1): 39.6% reduction with 15 μ M hydrogen peroxide, 22.7% reduction with 50 μ M glutamate treatment, 17.6% reduction with 75 nM rotenone, and a 25.7%, 38.1%, and 49% reduction with 5, 7.5, and 10 μ M A β ₁₋₄₂, respectively (Fig. 1). HU treatment significantly attenuated the reduction in cell viability by 20.5%, 17.5%, 13.5%, 9.8% and 11.2% for neurons treated with hydrogen peroxide, glutamate, rotenone, and 5 and 7.5 μ M A β ₁₋₄₂, respectively (Table 1). These data suggest HU treatment protects hippocampal neurons, to some extent, against increased oxidative stress, increased excitotoxicity, decreased mitochondrial function, and insults caused by A β aggregation and accumulation.

3.2 HU treatment attenuates H₂O₂-induced reductions in respiration measures

We chose to perform subsequent experiments with only one neurotoxic agent hydrogen peroxide. Hydrogen peroxide increases cellular oxidative stress, a component of multiple neurodegenerative diseases; and HU treatment attenuated the effects of hydrogen peroxide on cell viability to the greatest extent. Rotenone was specifically not chosen because it directly inhibits mitochondrial complex I function.

To determine the effects of HU treatment on neuronal energy metabolism, we measured cellular oxygen consumption rates (OCR) using the Seahorse XF-96 extracellular flux analyzer. HU treatment of hippocampal neurons did not significantly alter basal measurements of energy metabolism (Fig. 2A). To test whether HU treatment could instead precondition neurons against a toxic insult, we treated neurons with H₂O₂ (35 μ M). H₂O₂ treatment significantly decreased basal respiration, ATP-linked respiration, maximal respiration, mitochondrial reserve capacity, and non-mitochondrial respiration (Fig. 2A; One-way ANOVAs $p = 0.014$; *post-hoc* Bonferroni tests $p < 0.05$, $p < 0.05$, $p < 0.001$, $p < 0.01$, $p < 0.01$, respectively). HU treatment significantly attenuated the H₂O₂-induced reductions in maximal respiration, mitochondrial reserve capacity, and non-mitochondrial linked respiration (*post-hoc* Bonferroni tests $p < 0.05$). The mitochondrial reserve capacity is the difference between the ATP produced by oxidative phosphorylation at basal levels and that produced at maximal activity (Desler et al., 2012). The reserve is needed if a stressor, such as a neurotoxin, or an increased workload requires a sudden burst of energy. Notably, HU treatment did not attenuate H₂O₂-induced reductions in respiratory measures if basal levels of respiration in H₂O₂-treated neurons dropped below 50% of untreated control basal respiration levels. Together, these data suggest HU treatment improves the oxidative phosphorylation capacity in neurons under conditions of oxidative stress.

3.3 HU increases neuronal ATP content

HU treatment sustained maximal respiration, an ATP-dependent process, in neurons subjected to oxidative stress. Therefore, we measured the effect of HU on neuronal ATP content. HU treatment significantly increased neuronal ATP content compared to untreated control neurons and neurons treated with 15 μ M H₂O₂ for one hour (Fig. 2B; 2-way ANOVA $p_{H_2O_2} = 0.002$, $p_{HU} = 0.005$, *post-hoc* Bonferroni tests 15 μ M H₂O₂ vs 15 μ M H₂O₂ + HU $p < 0.05$).

3.4 HU treatment increases expression of mitochondrial and autophagy related proteins

We performed microarray gene expression analyses on cultured rat hippocampal neurons treated with HU for 2, 6, 12, and 24 hours to gain further insight into the neuroprotective mechanisms of HU. Gene ontology and pathway enrichment analyses comparing neurons treated with HU for 2 hours or 24 hours demonstrated an increase in mitochondrial- and proteasomal-related genes compared to untreated control neurons (Supplementary Table S1).

Microarray pathway analyses suggested components of adaptive cellular stress response pathways were upregulated in HU-treated neurons. We used western blotting to examine the expression of various subunits of the mitochondrial electron transport chain complexes, the autophagy related protein LC3, and the mitochondrial deacetylase SIRT3 in hippocampal

neurons treated with HU for 24 hours. HU treatment significantly increased expression of MTCO1, a cytochrome c oxidase subunit of mitochondrial electron transport complex IV ($p < 0.05$), and significantly increased total expression of the autophagy marker protein LC3 without affecting the ratio of LC3II/LC3I (Fig. 3A, B; $p < 0.05$). Although SIRT3 has been shown to mediate adaptive responses to oxidative and excitatory stress, HU treatment did not consistently increase SIRT3 protein expression (Fig. 3C). Since only expression of a subunit of mitochondrial complex IV, and not the markers of complexes I (NDUF8), II (SDHB), III (UQCRC2), and V (ATP5A), increased, HU treatment may increase oxidative phosphorylation capacity without increasing overall mitochondrial biogenesis.

Next, we assessed proteasome activity after HU treatment of wild type hippocampal neurons. *In vitro* measurements of 20S and 26S proteasome activities using cell lysates from neurons treated with HU for 2 or 24 hours indicated that HU treatment did not alter 20S or 26S proteasome activity (Fig. 4; One-way ANOVA $p_{20S} = 0.148$, $p_{26S} = 0.544$).

3.5 Impact of HU on behavior and A β pathology in APP/PS1 AD mice

Neuronal damage and memory loss are prominent features of AD (Association, 2016). Based on the ability of HU to attenuate the effects of hydrogen peroxide, glutamate, rotenone, and A β_{1-42} peptide in wild type rat hippocampal neurons, we tested the possible neuroprotective effects of HU in an animal model of AD. Two cohorts of APP/PS1 mice and age-matched non-transgenic (NTG) control mice were treated with HU starting between 2–5 months of age. Cohort 1 was treated for a total of five months and cohort 2 for nine months. We first performed behavioral experiments on the younger cohort, cohort 1. Based on these results, we aged the APP/PS1 mice longer before performing behavioral testing in cohort 2. We reasoned that the cognitive deficits in the aged APP/PS1 mice would be greater than in the younger mice; and therefore, the effects of HU treatment on cognitive measures may be more readily discernable. Behavioral testing began between 6–8 months of age for cohort 1 and between 10.5–13 months of age for cohort 2.

3.6 APP/PS1 mice did not exhibit anxiety-related behaviors

The open field test was used to assess locomotion and exploratory behavior and the elevated plus maze was used to assess anxiety-related behavior in the mice (Bailey and Crawley, 2009). Mice that spend more time in the center zone of the open field or more time in the open arms of the elevated plus maze are considered to exhibit less anxiety than mice who spend less time in the open areas. The 6 to 8-month-old APP/PS1 mice and HU-treated APP/PS1 mice traveled a significantly greater distance and spent significantly more time moving than the NTG control mice in the open field test (Fig. 5A-B; One-way ANOVA $p_{\text{distance}} = 0.006$, post-*hoc* Bonferroni tests $p < 0.05$; One-way ANOVA $p_{\text{time}} = 0.002$, post-*hoc* Bonferroni tests $p < 0.01$). However, there was no significant difference between groups of mice in the distance traveled or the time spent in the center zone. There were no significant differences in any of the open field test measurements between the 10 to 13-month-old NTG mice, APP/PS1 mice, or either HU-treated group of mice (Fig. 5C-D). Similarly, there were no significant differences in either cohort between NTG, APP/PS1, or HU-treated mice in the time spent in the open arms or the time spent in the closed arms and middle zone of the elevated plus maze (Fig. 6). Neither age group of APP/PS1 mice

exhibited anxiety-related behavior significantly different from NTG control mice in either behavioral test; and, HU-treatment did not significantly alter the behavior of mice in either test.

3.7 Hydroxyurea treatment improved spatial reference memory in APP/PS1 mice

The MWM was used to assess the effects of HU-treatment on spatial learning and memory in APP/PS1 mice, a hippocampus-dependent task (Vorhees and Williams, 2006). During the training trials, there was a significant difference in the escape latency in the younger cohort (cohort 1), but not in the older cohort (cohort 2). The younger NTG control mice found the platform in significantly less time than the younger APP/PS1 mice on the seventh day of training (Fig. 7A; RM-ANOVA $p = 0.032$, post-hoc Bonferroni NTG vs APP/PS1 day 7 $p < 0.05$). HU treatment reduced the time the younger APP/PS1 mice took to find the platform on the seventh day of training in cohort 1, but had no effect on the ability of the NTG or APP/PS1 mice to learn the location of the hidden platform in cohort 2 (Fig. 8A). Additionally, there were no significant differences in the distance swam to locate the platform or the average swim speed between any of the groups in cohorts 1 or 2 (Fig. 7B-C and 8B-C).

Twenty-four and 48 hours after the last training trials the hidden platform was removed to test the spatial reference memory of the mice. Spatial reference memory was analyzed by 1) examining the percentage of time swam in the quadrant where the platform was located during training, the target quadrant, compared to the chance level of 25%, 2) comparing the percentage of time swam in the target quadrant between groups, and 3) comparing the percentage of time swam in the target quadrant versus the non-target quadrants within groups. These data are summarized in Table 2. For cohort 1, NTG control and HU-treated APP/PS1 mice spent significantly more time in the target quadrant compared to the 25% chance level during the 24-hour and 48-hour probe trials (Fig. 7D and F). There were no statistical differences in the time spent in the target quadrant between groups during either probe trial. However, all three groups of younger mice, NTG control, APP/PS1, and HU-treated APP/PS1, spent significantly more time in the target quadrant compared to the non-target quadrants during the 24-hour probe trial (Fig. 7E). However, only the NTG control mice and HU-treated APP/PS1 mice spent significantly more time in the target quadrant compared to the time spent in each non-target quadrant during the 48-probe trial (Fig. 7G). This suggests HU treatment improved the spatial reference memory of the younger APP/PS1 mice.

Since the APP/PS1 mice spent significantly more time in the target quadrant than the non-target quadrants during the 24-hour probe trial, this suggested they remembered the location of the platform. Therefore, we decided to age the cohort 2 mice longer before behavioral testing to possibly increase their learning and memory deficits; and, we shorten the training period from nine days to six days for cohort 2. In the older cohort of mice (cohort 2), the NTG control mice and HU-treated NTG control mice spent significantly more time in the target quadrant compared to chance during the 24-hour probe trial (Fig. 8D); and the HU-treated NTG control mice and HU-treated APP/PS1 mice spent significantly more time in the target quadrant compared to chance during the 48-hour probe trial (Fig. 8F). During the

24-hour probe trials the NTG control mice spent significantly more time in the target quadrant compared to APP/PS1 mice (Fig. 8E), but not during the 48-hour probe trials (Fig. 8F). During the 24-hour and 48-hour probe trials, the older untreated APP/PS1 mice did not spent significantly more time in the target quadrant compared to the time spent in the three other non-target quadrants (Fig. 8E and 8G). However, the older HU-treated APP/PS1 mice spent significantly more time in the target quadrant than in the lower right or lower left quadrants during the 24-hour probe trials (Fig. 8E) and spent significantly more time in the target quadrant compared to all three non-target quadrants during the 48-hour probe trial (Fig. 8G). Taken together, these data suggest that HU-treatment significantly improved the spatial reference memory of APP/PS1 mice in both the younger and older mice.

Analyses of thigmotaxic behavior, the tendency of some mice to swim around the perimeter of the pool, indicated that the younger APP/PS1 mice swam a significant amount of time in the perimeter of the pool during the 24-hour probe trial and that HU-treatment significantly reduced thigmotaxic behavior in the younger APP/PS1 mice (Fig. 7H; One-way ANOVA $p = 0.002$, *posthoc* Bonferroni analyses p_{WT} vs APP/PS1 < 0.01 , $p_{APP/PS1}$ vs APP/PS1 + HU < 0.05). The older APP/PS1 mice exhibited significant thigmotaxic behavior compared to the NTG control mice (Fig. 8H; One-way ANOVA $p = 0.064$, *post-hoc* Bonferroni's p_{NTG} vs. APP/PS1 < 0.05). However, in cohort 2 thigmotaxic behavior in the HU-treated APP/PS1 mice did not significantly differ from the APP/PS1 mice or NTG control mice. As a cued control, the task was performed with a visible platform; and, there were no significant differences in the latency to find a visible platform between any of the groups ($p_{cohort 1} = 0.589$, $p_{cohort 2} = 0.101$). In summary, HU-treatment decreased thigmotaxic behavior in the younger APP/PS1 mice.

3.8 Hydroxyurea treatment did not alter soluble A β levels

We measured soluble A β_{42} and A β_{40} levels in cortical and hippocampal samples from the younger (cohort 1) APP/PS1 and HU-treated APP/PS1 mice (Fig. 9). HU treatment did not significantly alter soluble A β_{42} and A β_{40} levels or the ratio of A β_{42} /A β_{40} peptide levels in either brain tissue. However, there was a trend toward higher soluble levels of A β_{42} and A β_{40} in HU-treated APP/PS1 hippocampal samples ($p = 0.063$ and $p = 0.066$, respectively).

3.9 Microarray analyses of gene expression in mouse hippocampi

To gain insight into the molecular mechanisms that underlie improved spatial reference memory in APP/PS1 mice following HU treatment, we performed microarray analyses on hippocampi isolated from NTG control mice and NTG control mice treated with HU for two and a half months. NTG control mice were treated so that the findings could potentially be applicable to other neurodegenerative diseases. Gene ontology (GO) and pathway enrichment analyses identified upregulation of multiple GO groups related to synaptic function and adaptive cellular stress responses including mitochondrial electron transport function, the lysosome, the heat shock response and unfolded protein response (GO0051082), and the antioxidant response (Table 3 and Supplementary Table S2). Further work will be necessary to confirm these data. This includes examining gene expression in hippocampi from HU-treated APP/PS1 mice.

4. Discussion

In this study, we found that HU provides neuroprotection *in vitro* against neurotoxins that increase oxidative stress, increase excitotoxicity, reduce mitochondrial function, and mimic aspects of AD (exposure to aggregating A β). *In vivo* we demonstrated that HU treatment improves spatial reference memory in APP/PS1 mice independent from a reduction in soluble A β levels. Although we have not fully established the mechanism(s) by which HU provides neuroprotection, our primary neuronal data suggests HU increases oxidative phosphorylation capacity as evidenced by an increase in maximal respiration, mitochondrial reserve capacity, and ATP content in neurons treated with hydrogen peroxide. We further found that HU treatment results in increased levels of mitochondrial electron transport chain complex proteins and increases levels of LC3, a protein involved in autophagy. Microarray gene expression analyses of HU-treated wild type rat hippocampal neurons and hippocampal tissue from HU-treated NTG control mice further supported these findings and suggest that HU increases mitochondrial gene expression.

Studies of AD patients, and cell culture and animal models of AD, have provided evidence for the involvement of mitochondrial dysfunction (Mattson et al., 2008), oxidative stress (Texel and Mattson, 2011), excitotoxicity (Camandola and Mattson, 2011) and impaired lysosome function and autophagy (Kerr et al., 2017; Nixon, 2013), in the neurodegenerative process. Aggregating A β can trigger neuronal degeneration by inducing membrane lipid peroxidation which results in the impairment of membrane ion-motive ATPases, and glutamate and glucose transporters, thereby rendering neurons vulnerable to excitotoxicity (Keller et al., 1997; Mark et al., 1995; Mark et al., 1997; Mattson et al., 1992). We found that HU pretreatment protects primary hippocampal neurons against multiple insults involved in A β toxicity, results consistent with a hormesis-based mechanism that up-regulates multiple adaptive cellular stress response pathways (Calabrese and Mattson, 2017). HU is best known as a ribonucleotide reductase inhibitor that suppresses DNA synthesis and, by that mechanism, can prevent aberrant cell proliferation in patients with chronic myeloproliferative disorders and some types of cancer (Spivak and Hasselbalch, 2011). However, neurons are postmitotic, do not express ribonucleotide reductase (Zhu et al., 2003), and do not replicate their nuclear DNA. Therefore, the neuroprotective mechanism of action of HU is likely not the result of induction of replicative stress.

A major finding from the behavioral tests of the present study was that the deficit in memory retention in the water maze probe trials was ameliorated in HU-treated APP/PS1 mice compared to control APP/PS1 mice. This beneficial effect of HU on cognition was apparently not the result of an effect on the accumulation of A β in the brain because the concentrations of soluble A β ₄₂ and A β ₄₀ were unaffected by HU treatment. Previous studies have provided evidence that A β impairs synaptic function by mechanisms involving oxidative stress (Hensley et al., 1996; Texel and Mattson, 2011), mitochondrial dysfunction (Keller et al., 1997; Ma et al., 2011; Reddy and Beal, 2008), and impaired autophagy (Kerr et al., 2017; Nixon, 2007). The ability of HU to protect cultured hippocampal neurons against A β toxicity, and oxidative, metabolic and excitotoxic stress, suggests that HU may protect synapses and ameliorate learning and memory deficits by suppressing oxidative, metabolic and excitotoxic stress.

It is known that HU crosses the blood-brain barrier (Dogruel et al., 2003; Syvanen et al., 2007). A potential mechanism by which HU increases neuronal stress resistance in the brain is suggested by evidence that HU can induce a moderate elevation of intracellular Ca^{2+} levels and thereby increase the production of nitric oxide, which then activates soluble guanylate cyclase to generate cyclic GMP (Almeida et al., 2012; Cokic et al., 2006; Raththagala et al., 2010). Previous studies have shown that cyclic GMP can protect neurons against excitotoxicity, oxidative stress and A β toxicity (Barger et al., 1995; Keller et al., 1998; Orio et al., 2007). Nitric oxide and cyclic GMP also play important roles in synaptic plasticity and learning and memory (Hawkins et al., 1998; Prieto et al., 2017; Wincott et al., 2014). Future studies will be required to establish whether calcium- and NO-mediated signaling mediate the neuroprotective and cognition-preserving effects of HU documented in the primary neuronal culture and APP/PS1 experiments of the present study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Hydroxyurea treatment protects hippocampal neurons against neurotoxic stress
- Hydroxyurea attenuates reductions in mitochondrial respiration induced by H_2O_2
- Hydroxyurea increases neuronal ATP content
- HU treatment increases expression of mitochondrial and autophagy related proteins
- Hydroxyurea improves reference memory in the APP/PS1 Alzheimer's mouse model

Figure 1. MTS viability assays.

HU treatment of rat hippocampal neurons significantly attenuates the significant reduction in cell viability induced by: A) hydrogen peroxide (RM-ANOVA $p_{H_2O_2} = 0.0001$, $p_{HU} = 0.0007$, *post-hoc* Bonferroni tests CON vs HU and HU + 15 μM H_2O_2 vs 15 μM H_2O_2 $p < 0.05$; $n = 6$); B) glutamate (RM-ANOVA $p_{GLUT} = 0.002$, $p_{HU} = 0.004$, *post-hoc* Bonferroni tests CON vs HU and HU + 50 μM glutamate vs 15 μM glutamate $p < 0.05$; $n = 9$); C) rotenone (RM-ANOVA $p_{ROT} = 0.05$, $p_{HU} = 0.011$, *post-hoc* Bonferroni tests HU + 75 μM rotenone vs 75 μM rotenone $p < 0.01$; $n = 3$); and D) $A\beta_{1-42}$ peptide (RM-ANOVA $p_{A\beta} = 0.0001$, $p_{HU} = 0.0001$, *post-hoc* Bonferroni tests HU + 5 μM $A\beta_{1-42}$ and HU + 7.5 μM $A\beta_{1-42}$ vs 5 μM and 7.5 μM $A\beta_{1-42}$ $p < 0.05$; $n = 5$). * $p < 0.05$, ** $p < 0.01$. CON = untreated control neurons. Errors bars = SEM.

Figure 2. Mitochondrial measures.

A) Relative oxygen consumption rates (OCR). HU treatment significantly attenuated the significant reduction in maximal respiration, energetic reserve capacity, and non-mitochondrial respiration caused by H₂O₂ treatment (35 μM; One-way ANOVAs within measurements $p = 0.014$, *post-hoc* Bonferroni tests CON vs H₂O₂ & $p < 0.05$, $p < 0.01$, $p < 0.001$, HU vs HU/H₂O₂ * $p < 0.05$). B) Relative ATP content. The significant reduction in ATP content by hydrogen peroxide treatment of hippocampal neurons was significantly attenuated by HU treatment (2-way ANOVA $p_{H_2O_2} = 0.002$, $p_{HU} = 0.005$, *post-hoc* Bonferroni tests 15 μM H₂O₂ vs 15 μM H₂O₂ + HU * $p < 0.05$). CON = untreated control neurons. Errors bars = SEM, $n = 3$ independent experiments.

Figure 3. Western blot analyses of mitochondrial and autophagy related protein expression.

A) Western blot analyses of mitochondrial electron transport chain subunits (Complex I-V, CI-CV). Complex IV subunit MTCO1 significantly increased after 16 hours of HU treatment. B) Expression of LC3-I and LC3-II significantly increased after 24 hours of HU treatment. C) SIRT3 expression did not significantly increase after HU treatment. Relative protein expression normalized to untreated control neuron (CON) expression. Errors bars = SEM, n = 3 independent experiments. * $p < 0.05$, ** $p < 0.01$.

Figure 4. Proteasome activity.

HU treatment did not significantly alter the activity of the 20S or 26S proteasome in rat hippocampal neurons (One-way ANOVA $p_{20S} = 0.148$, $p_{26S} = 0.544$, $n = 4$). CON = untreated control neurons. Errors bars = SEM.

Figure 5. Open Field Test.

Six to eight-month-old APP/PS1 mice exhibit more hyperactivity in the open field test than non-transgenic (NTG) control mice. 6 to 8-month-old mice (A-D). NTG $n = 11$, APP/PS1 and APP/PS1 + HU $n = 7$. A) Total distance traveled (One-way ANOVA $p = 0.006$, post-*hoc* Bonferroni tests * $p < 0.05$). B) Total ambulatory time (One-way ANOVA $p = 0.002$, post-*hoc* Bonferroni tests ** $p < 0.01$). C) Total distance traveled in the center zone ($p = 0.140$). D) Total time spent in the center zone ($p = 0.151$). 10 to 13-month-old mice (E-H). NTG $n = 5$, NTG + HU $n = 8$, APP/PS1 $n = 13$, APP/PS1 + HU $n = 12$. E) Total distance traveled ($p = 0.468$). F) Total ambulatory time ($p = 0.119$). G) Total distance traveled in the center zone ($p = 0.836$). H) Total time spent in the center zone ($p = 0.267$). Errors bars = SEM.

Figure 6. Elevated plus maze.

HU treatment did not affect performance in the elevated plus maze. 6 to 8-month-old mice (A-B). NTG $n = 11$, APP/PS1 $n = 6$, and APP/PS1 + HU $n = 7$. A) Time in the open arms ($p = 0.901$). B) Time in the closed arms and middle zone ($p = 0.782$). 10 to 13-month-old mice (C-D). NTG $n = 9$, NTG + HU $n = 8$, APP/PS1 $n = 13$, APP/PS1 + HU $n = 13$. C) Time in the open arms ($p = 0.782$). D) Time in the closed arms and middle zone ($p = 0.147$). Errors bars = SEM.

Figure 7. Morris water maze cohort 1.

HU treatment improved spatial reference memory in 6 to 8-month-old APP/PS1 mice. A) Latency to reach the hidden platform across training days. There was a significant difference among groups in their latency to find the hidden platform. The NTG control mice found it in significantly less time than APP/PS1 mice on Day 7 (2-way RM-ANOVA $p = 0.032$, *post-hoc* Bonferroni analyses NTG vs APP/PS1 Day 7 $*p < 0.05$). B) Distance to reach the hidden platform across training days. C) Average swim speed across training days. There was no significant difference between groups in the distance traveled to reach the hidden platform or the group swim speeds. D) 24-hour probe trial time in target quadrant. NTG and HU-treated APP/PS1 mice spent significantly more time in the target quadrant compared to the 25% chance level (One-way ANOVA $p = 0.007$, *post-hoc* Bonferroni analyses $p_{\text{NTG}} < 0.01$ and $p_{\text{APP/PS1 + HU}} < 0.05$). There was no statistical difference between groups (One-way ANOVA $p = 0.634$). E) 24-hour probe trial quadrant preference. NTG control, APP/PS1, and HU-treated APP/PS1 mice all spent significantly more time in the upper right target quadrant than in the non-target quadrants (UL = upper left, LR = lower right, LL = lower left; one way ANOVAs within groups, $p_{\text{NTG}} = 0.0001$, $p_{\text{APP/PS1}} = 0.005$, $p_{\text{APP/PS1 + HU}} = 0.001$, *post-hoc* Dunnett's multiple comparison tests $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). F) 48-hour probe trial time in target quadrant. NTG and HU-treated APP/PS1 mice spent significantly more time in the target quadrant compared to the 25% chance level (One-way ANOVA $p = 0.023$, *post-hoc* Bonferroni analyses $p < 0.05$). There was no statistical difference between groups (Oneway ANOVA $p = 0.257$). G) 48-hour probe trial quadrant preference. NTG and HU-treated APP/PS1 mice spent significantly more time in the target quadrant than in the non-target quadrants (One way ANOVA, $p_{\text{NTG}} = 0.001$, $p_{\text{APP/PS1}} = 0.443$, $p_{\text{APP/PS1 + HU}} = 0.002$, *post-hoc* Dunnett's multiple comparison

tests * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). H) Thigmotaxis. HU-treatment significantly reduced thigmotaxis in APP/PS1 mice during the 24-hr probe trial (One way ANOVA $p = 0.002$, *post-hoc* Bonferroni analyses $p_{WT \text{ vs APP/PS1}} < 0.01$, $p_{APP/PS1 \text{ vs APP/PS1 + HU}} < 0.05$). NTG $n = 11$, APP/PS1 $n = 7$, APP/PS1 + HU $n = 7$. Errors bars = SEM.

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Figure 8. Morris water maze cohort 2.

HU-treatment improved spatial reference memory in 10 to 13-month-old APP/PS1 mice. A) Latency to reach the hidden platform. B) Distance to reach the hidden platform. C) Average swim speed. There was no significant difference in escape latency, distance to find the platform, or swim speed between the four groups of mice during the six-day training period (2-way RM-ANOVA $p = 0.533$, $p = 0.973$, and $p = 0.601$, respectively). D) 24-hour probe trial time in target quadrant. NTG and HU-treated NTG mice spent significantly more time in the target quadrant compared to the 25% chance level (One-way ANOVA $p = 0.0003$, *post-hoc* Bonferroni analyses $p_{\text{NTG}} < 0.001$ and $p_{\text{NTG + HU}} < 0.01$). NTG mice spent significantly more time than APP/PS1 mice in the target quadrant (One-way ANOVA $p = 0.021$, *post-hoc* Bonferroni analyses NTG vs APP/PS1 $**p < 0.01$). E) 24-hour probe trial quadrant preference. NTG control, HU-treated NTG control, and HU-treated APP/PS1 mice spent significantly more time in the upper right (UR) target quadrant than in non-target quadrants (UL = upper left, LR = lower right, LL = lower left; one-way ANOVAs within groups NTG, NTG + HU, and APP/PS1 + HU $p = 0.0001$, $p_{\text{APP/PS1}} = 0.089$, *post-hoc* Dunnett's multiple comparison tests $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). F) 48-hour probe trial time in target quadrant. HU-treated NTG mice and HU-treated APP/PS1 mice spent significantly more time in the target quadrant compared to the 25% chance level (One-way ANOVA $p = 0.023$, *post-hoc* Bonferroni analyses $p < 0.05$). G) 48-hour probe trial quadrant preference. NTG control, HU-treated NTG control, and HU-treated APP/PS1 mice spent significantly more time in the upper right target quadrant than in the non-target quadrants (One-way ANOVA within group $p_{\text{NTG}} = 0.012$, $p_{\text{NTG + HU}} = 0.001$, $p_{\text{APP/PS1}} = 0.221$, $p_{\text{APP/PS1 + HU}} = 0.001$, *post-hoc* Dunnett's multiple comparison tests $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). H) 24-hr probe thigmotaxis. APP/PS1 mice exhibited significantly more

thigmotaxis than NTG mice (One way ANOVA $p = 0.064$, *post-hoc* Bonferroni analyses NTG vs APP/PS1 $p < 0.05$). NTG $n = 9$, NTG + HU $n = 8$, APP/PS1 $n = 13$, APP/PS1 + HU $n = 13$. Bars = SEM.

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Figure 9. Aβ levels in cohort 1 mouse brains.

Aβ₄₂ and Aβ₄₀ peptide levels were measured in brain homogenates from the A) cortex and B) hippocampus of APP/PS1 (n = 7) and APP/PS1 + HU (n = 8) mice in cohort 1. There were no significant differences between Aβ₄₂ and Aβ₄₀ levels in the cortical (p = 0.224) or hippocampal (p = 0.063) samples or between the ratio of Aβ₄₂/Aβ₄₀ (C; p = 0.415) in the cortical or hippocampal samples.

Table 1.

Summary of MTS viability assay data.

Toxin	Sample size (n)	RM-ANOVA p-value _{toxin}	<i>Post-hoc</i> Bonferroni p-value _{HU+toxin vs toxin alone}
15 μ M H ₂ O ₂	6	p = 0.0001	p < 0.05
50 μ M glutamate	9	p = 0.002	p < 0.05
75 μ M rotenone	3	p = 0.011	p < 0.01
5 μ M A β ₁₋₄₂	5	p = 0.0001	p < 0.05
7.5 μ M A β ₁₋₄₂	5	p = 0.0001	p < 0.05

Table 2.
Summary of statistical analyses for Morris water maze probe trials.

Cohort 1: NTG n = 11, APP/PS1 n = 7, APP/PS1 + HU n = 7. Cohort 2: NTG n = 9, NTG + HU n = 8, APP/PS1 n = 13, APP/PS1 + HU n = 13.

Measurement	Cohort	Probe trial	One-way ANOVA	post-hoc p-value
Percent time in target quadrant compared to chance level (25%)	1	24-hr	p = 0.007	p _{NTG} < 0.01 p _{APP/PS1+HU} < 0.05
	1	48-hr	p = 0.023	p _{NTG} < 0.05 p _{APP/PS1 + HU} < 0.05
	2	24-hr	p = 0.0003	p _{NTG} < 0.001 p _{NTG + HU} < 0.01
	2	48-hr	p = 0.034	p _{NTG + HU} < 0.05 p _{APP/PS1 + HU} < 0.05
Percent time in target quadrant compared between groups	1	24-hr	p = 0.634	
	1	48-hr	p = 0.257	
	2	24-hr	p = 0.021	p _{NTG vs APP/PS1} < 0.01
	2	48-hr	p = 0.353	
Percent time spent in target quadrant vs non-target quadrants	1	24-hr	p _{NTG} = 0.001 p _{APP/PS1} = 0.005 p _{APP/PS1 + HU} = 0.001	NTG: p _{UR vs UL} , p _{LR, LL} < 0.001 APP/PS1: p _{UR vs LR, LL} < 0.01, p _{UR vs UL} < 0.05 APP/PS1+HU: p _{UR vs UL, LR, LL} < 0.001
	1	48-hr	p _{NTG} = 0.001 p _{APP/PS1 + HU} = 0.001	NTG: p _{UR vs UL} < 0.05, p _{LR, LL} < 0.001 APP/PS1+HU: p _{UR vs UL, LR, LL} < 0.001
	2	24-hr	p _{NTG} = 0.0001 p _{NTG + HU} = 0.001 p _{APP/PS1 + HU} = 0.001	NTG: p _{UR vs UL} , p _{LR, LL} < 0.001 NTG + HU: p _{UR vs LR, LL} < 0.001, p _{UR vs UL} < 0.01 APP/PS1 + HU: p _{UR vs LR, LL} < 0.001

Table 3.
Gene ontology analysis of genes altered at the mRNA level by HU treatment of wild type mice.

Microarray analyses were performed on wild type mice treated with HU (45 mg/kg/day) for 2.5 months. GO term analysis identified the pathways above as upregulated by HU treatment. N=3 untreated wild type mice, n = 3 HU-treated wild type mice.

Gene Ontology Term	Z_Score
GO0007608 SENSORY PERCEPTION OF SMELL	10.72
GO0004984 OLFACTORY RECEPTOR ACTIVITY	10.71
GO0004930 G PROTEIN COUPLED RECEPTOR ACTIVITY	9.34
GO0007186 G PROTEIN COUPLED RECEPTOR PROTEIN SIGNALING PATHWAY	9.24
GO0004871 SIGNAL TRANSDUCER ACTIVITY	8.51
GO0007165 SIGNAL TRANSDUCTION	7.00
GO0004872 RECEPTOR ACTIVITY	6.42
GO0031424 KERATINIZATION	6.09
GO0001533 CORNIFIED ENVELOPE	5.03
GO0003735 STRUCTURAL CONSTITUENT OF RIBOSOME	4.67
GO0005840 RIBOSOME	4.63
GO0006412 TRANSLATION	4.61
GO0016020 MEMBRANE	4.45
GO0030529 RIBONUCLEOPROTEIN COMPLEX	4.40
GO0016021 INTEGRAL TO MEMBRANE	4.08
GO0005179 HORMONE ACTIVITY	3.70
GO0005230 EXTRACELLULAR LIGAND GATED ION CHANNEL ACTIVITY	3.33
GO0005843 CYTOSOLIC SMALL RIBOSOMAL SUBUNIT	3.23
GO0005739 MITOCHONDRION	3.13
GO0050896 RESPONSE TO STIMULUS	3.08
GO0016469 PROTON TRANSPORTING TWO SECTOR ATPASE CO	3.07
GO0008137 NADH DEHYDROGENASE (UBIQUINONE) ACTIVITY	2.98
GO0046933 HYDROGEN ION TRANSPORTING ATP SYNTHASE ACTIVITY	2.93
GO0046961 HYDROGEN ION TRANSPORTING ATPASE ACTIVITY	2.93
GO0042254 RIBOSOME BIOGENESIS AND ASSEMBLY	2.91
GO0006754 ATP BIOSYNTHETIC PROCESS	2.91
GO0005830 CYTOSOLIC RIBOSOME (SENSU EUKARYOTA)	2.89
GO0045202 SYNAPSE	2.85
GO0015986 ATP SYNTHESIS COUPLED PROTON TRANSPORT	2.74
GO0015078 HYDROGEN ION TRANSMEMBRANE TRANSPORTER ACTIVITY	2.73
GO0060113 INNER EAR RECEPTOR CELL DIFFERENTIATION	2.60
GO0009887 ORGAN MORPHOGENESIS	2.58
GO0005743 MITOCHONDRIAL INNER MEMBRANE	2.44

Gene Ontology Term	Z_Score
GO0003924 GTPASE ACTIVITY	2.36
GO0008021 SYNAPTIC VESICLE	2.31
GO0001824 BLASTOCYST DEVELOPMENT	2.24
GO0045211 POSTSYNAPTIC MEMBRANE	2.19
GO0005842 CYTOSOLIC LARGE RIBOSOMAL SUBUNIT	2.16
GO0005764 LYSOSOME	2.12
GO0003954 NADH DEHYDROGENASE ACTIVITY	2.10
GO0007269 NEUROTRANSMITTER SECRETION	2.04
GO0004129 CYTOCHROME C OXIDASE ACTIVITY	2.01
GO0043234 PROTEIN COMPLEX	1.98
GO0030672 SYNAPTIC VESICLE MEMBRANE	1.97
GO0008180 SIGNALOSOME	1.95
GO0015935 SMALL RIBOSOMAL SUBUNIT	1.86
GO0016616 OXIDOREDUCTASE ACTIVITY ACTING ON THE CH-OH GROUP OF DONORS	1.84
GO0005765 LYSOSOMAL MEMBRANE	1.80
GO0051082 UNFOLDED PROTEIN BINDING	1.80
GO0005905 COATED PIT	1.79
GO0045261 PROTON TRANSPORTING ATP SYNTHASE COMPLEX	1.79
GO0045104 INTERMEDIATE FILAMENT CYTOSKELETON ORGAN	1.79
GO0017157 REGULATION OF EXOCYTOSIS	1.77
GO0008199 FERRIC IRON BINDING	1.76
GO0003723 RNA BINDING	1.74
GO0006120 MITOCHONDRIAL ELECTRON TRANSPORT NADH TO UBIQUINONE	1.70
GO0042734 PRESYNAPTIC MEMBRANE	1.66
GO0019717 SYNAPTOSOME	1.65
GO0019843 RRNA BINDING	1.64
GO0045263 PROTON TRANSPORTING ATP SYNTHASE COMPLEX	1.59
GO0043005 NEURON PROJECTION	1.58
GO0006099 TRICARBOXYLIC ACID CYCLE	1.57
GO0015031 PROTEIN TRANSPORT	1.51