

A Dietary Supplement Abolishes Age-Related Cognitive Decline in Transgenic Mice Expressing Elevated Free Radical Processes

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We previously found that transgenic mice overexpressing growth hormone (TGM) have elevated and progressively increasing free radical processes in brain that strongly correlates with reduced survivorship. Young mature TGM, however, displayed vastly enhanced learning of an eight-choice cued maze and qualitatively different learning curves than normal controls. Here we document the age-related patterns in learning ability of TGM and normal mice. Learning appeared inferior in both genotypes of very young mice but TGM were confirmed to be superior to normal mice upon maturity. Older TGM, however, showed rapid age-related loss of their exceptional learning, whereas normal mice at 1 year of age showed little change. The cognitive decline of TGM was abolished by a complex “anti-aging” dietary supplement formulated to promote membrane and mitochondrial integrity, increase insulin sensitivity, reduce reactive oxygen and nitrogen species, and ameliorate inflammation. Results are discussed in the context of reactive oxygen and nitrogen species, long-term potentiation, learning, aging and neuropathology, based on known impacts of the growth hormone axis on the brain, and characteristics of TGM. Exp Biol Med 228:800–810, 2003

Key words: aging; reactive oxygen and nitrogen species; learning; memory; long-term potentiation; neuronal signal transduction; transgenic growth hormone mice; growth hormone; insulin-like growth factor; cognitive decline

Age-related cognitive deterioration varies widely among individuals, suggesting that intervention is possible (1–4). Promising approaches include antioxidants and anti-inflammatories (5), dietary restriction (6, 7), growth factor augmentation (2), and stem cell manipu-

lations (8, 9). Transgenic animals with alterations of the growth hormone axis are promising models of brain structure, function, and age-related changes relevant to all of these interventions.

Hypothalamic somatostatin (SRIF) and growth hormone-releasing hormone (GHRH) regulate pituitary synthesis and secretion of growth hormone (GH), which in turn stimulates insulin-like growth factor-I (IGF-I) expression in many tissues and secretion from the liver (10, 11). Interventions to offset the reliable declines in GH and IGF-I that occur during mammalian aging ameliorate some symptoms of senescence (12). Both are antiapoptotic (i.e., neuroprotective) (2, 13, 14) and IGF-I regulates neurogenesis and regeneration, glial cell proliferation, myelination, neurite outgrowth, neuronal excitability, synaptic plasticity, and other growth factors (2, 15, 16). Whether activity of the GH axis offsets or exacerbates aging, however, remains controversial (17).

Remarkably, 50% of young mature transgenic growth hormone mice (TGM) learned an eight-choice cued maze before a single normal control (18). All TGM learned the task within 24 trials, whereas 30% of normal mice did not. TGM displayed an exponentially declining error rate whereas that of normal mice was linear (18). We and our colleagues showed TGM to be hypoactive (19), with greatly increased sleep, altered EEG patterns (20, 21), and altered feeding behavior (22). Others showed that TGM have inferior performance on aversive learning tasks (23), altered preferences for nicotine and ethanol (24), increased activity in open field tests, and higher sensitivity to amphetamine (35, 36).

The free radical theory of aging postulates that endogenously generated reactive oxygen and nitrogen species (RONS) cause accumulating damage to cellular lipids, proteins, and nucleic acids. Mitochondrial damage may engage an escalating cycle of increasing free radical generation and further damage (27–30). Damage may also induce inflammation and further generation of RONS by immunocytes and microglia (5). TGM have highly elevated and progressively increasing levels of lipid peroxidation and superoxide

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radical, particularly in the brain, where levels of superoxide radical and lipid peroxidation strongly correlate with longevity (31). A meta-analysis of the rodent literature confirmed that intraspecific longevity significantly declines with higher growth rates and larger mature body sizes in both rats and mice (17).

TGM express pathological changes in several organs consistent with elevated RONS (e.g., liver, kidney, heart) and that resemble senescent features of old rodents (e.g., Refs. 32, 33). The reduced longevity of TGM was consistent with their larger mass according to our meta-analysis (17). Thus, TGM at the very least represent a model of elevated free radical processes and pathologies resembling accelerated aging. Models of extended longevity are considered preferable for studying aging, but those expressing alterations in free radical generation, antioxidant defenses, and/or other processes related to aging also contribute valuable understanding (e.g., Refs. 34–36). Delayed cognitive aging occurs in dietary restricted rodents and dwarf mice characterized by small body size and reduced (but sometimes extended) GH axis activity (6, 37, 38). TGM appear dichotomously opposite to dietary restricted and dwarf rodents (17, 18), highlighting the need to know how their exceptional cognitive abilities change with age. Here we report that TGM show rapid loss of cognitive abilities with age.

Growth factors, including GH, IGF-1, and insulin, activate cellular transduction pathways that generate and require RONS as mediators of signal transduction. Conversely, RONS (e.g., nitric oxide [NO], superoxide radical [SOR], and hydrogen peroxide [H₂O₂]) mimic growth factor receptor ligands in activating the mitogen-activated protein kinase (MAPK/ERK) and phosphatidylinositol-3 kinase (PI3K) pathways (17). Across broad phylogenies genetic alterations affecting aging mainly involve the PI3K pathway (39). PI3K critically regulates glucose transport, antioxidant expression and apoptosis, and shows severe resistance to insulin signaling in TGM (40).

RONS and activation of MAPK/ERK (and perhaps PI3K) in neurons are essential for induction of long-term potentiation (LTP), long-term memory, and neuronal survival and development. RONS modulate neurotransmitter release, retrograde signaling to post-synaptic neurons during LTP and non-synaptic inter-cellular signaling between neurons and other brain cells (2, 41–43). Alternatively, RONS are implicated in cognitive aging and neuropathologies (e.g., Alzheimer's, Parkinson's, and Huntington's diseases, Down's syndrome, ALS, diabetic neuropathies, and ischemia reperfusion injuries; e.g., Refs. 5, 27, 44, 45). Such linkages suggest that both the early enhancement of cognition and its subsequent rapid decline in TGM may involve RONS processes.

With notable exceptions (e.g., Refs. 27–29, 46–50), supplements intended to ameliorate oxidative stress, inflammation, or associated manifestations of aging or neuropathology have yielded inconsistent or poor results. Further-

more, cocktails popularly self-administered by the public have had scant scientific scrutiny (5, 51). Most studies have tested one or at most, a few materials in combination. These materials commonly have synergistic or recycling interactions, however, that cannot be addressed by single-factor experiments (e.g., Refs. 27, 48, 51, 52).

Developing a complex supplement by adding and testing one material at a time could require inordinate time given the potential clinical value of an effective intervention. Consequently, we designed a complex anti-aging supplement (AASUP) based on known efficacy of ingredients to reduce RONS and inflammation, promote membrane and mitochondrial integrity and increase insulin sensitivity (all features strongly associated with aging and age-related pathologies). The AASUP contains 31 ingredients, including several recently highlighted as highly effective in combination (27). A large cohort of mice has received the AASUP daily for nearly three years. The AASUP not only abolished the age-related cognitive decline seen in TGM, but older treated mice learned a cued spatial maze in even fewer trials than younger TGM.

Materials and Methods

Animals. Our TGM mice have metallothionein promoters fused to rat GH structural genes (53). The rat GH genes are incorporated into one chromosome, chronically elevating plasma GH levels more than 100 fold. The original stock was C57BL/6J male × SJL female hybrids, donated by Dr. R. Brinster. The GH transgenes are inherited in Mendelian proportions so breeding normal females to heterozygously transgenic males yields equal numbers of normal and transgenic mice with otherwise similar genetic backgrounds. TGM were distinguishable by their larger size by 28 days of age. All mice were checked for cataracts and visual responsiveness to the experimenter. All mice were able to fully traverse the maze, although some untreated older TGM were slower than other mice.

General Housing Protocols. Four mice were maintained per cage (27 × 12 × 15.5 cm) bedded with wood-chip. A stainless steel hopper provided food *ad libitum* (LabDiet® 5001, PMI Feeds) and supported a water bottle. The housing room maintained a 12:12 h light:dark photoperiod at 22 ± 2°C. All protocols adhered to the Canada Council guidelines on animal care.

Apparatus. The apparatus was a cued variant of the 8-arm radial maze commonly employed in rodent learning research (18), but lacked arms. A circular arena was constructed from a 25-cm high barrier of flexible plastic (60 cm in diameter). This was surrounded by eight inverted plastic flower pots (15 cm in diameter) placed equidistantly around the outside of the barrier. A 5 cm × 5 cm door cut through the rim of each pot and aligned with similar holes in the barrier allowed mice access to pots from the central arena. The apparatus was set upon a plastic sheet covered with clean paper.

A number from 1 to 8 was affixed on the top of each inverted pot, and the entire apparatus was videotaped using an overhead camera. Discriminatory cues consisting of white circles or black squares (5 cm wide) were mounted above the doorways of even- or odd-numbered pots, respectively. The doorways were also outlined in black or white to correspond with the color of the associated shapes to augment discriminatory cues.

Cues were respectively paired with Petri dishes baited with a small dab of peanut butter that was either available or not. Accessible Petri dishes had a large hole cut through their lid, whereas the inaccessible dishes had numerous small holes pierced in the lid that allowed olfaction but prevented access to the food. The pairing of available food with either white or black discriminatory cues was randomly varied among the mice. Although most young mice learned the maze within 24 trials (18), 39 trials was chosen as a cut-off point for the present study because old mice often required more than 30 trials to learn. For each trial fresh food and Petri dishes were used, the paper floor was replaced, and the entire apparatus was washed and swabbed with 95% ethanol.

Thirty-two female normal mice (age range: 73 days to 892 days), 32 female TGM (age range: 48 to 452 days), and 24 supplemented female TGM (age range: 122 to 424 days) were tested. Mice were isolated 3 days before testing and fed small amounts of peanut butter to offset potential novelty responses to this food. Mice were fasted 1 h before testing to ensure motivation to eat. A perfect run in the maze consisted of the mouse entering each pot with available food only once while not entering any pots with inaccessible food. This is a difficult task for mice, so for the present study the task was considered to have been learned when, in three successive trials, the mouse made no more than a total of two errors. Two blocks of trials were run per day. Each block consisted of three trials presented sequentially with 10-min intervals between each trial. Five hours separated each block. All trials were conducted in the photophase.

Behavioral Scoring. Behavior was monitored and scored from videotapes. If the rear paws crossed the doorway threshold, a mouse was considered to have entered or exited a pot. Types of errors were: 1) wrong entry: entering a pot with inaccessible food; 2) wrong re-entry: re-entering a pot with screened food; and 3) correct re-entry: re-entering a pot with previously available food. All types of errors were summed to yield total errors.

Mature Mass. Mature mass was obtained from animals in the breeding colonies that had complete lifetime records. Male mature mass was considered the maximum achieved since older animals often lost weight. For females, mass during inter-pregnancy periods was used. Mature mass was analyzed using analysis of variance (ANOVA) and a Newman Keuls test.

Dietary Supplement. The AASUP was designed to simultaneously ameliorate several processes implicated in

aging (see Introduction). Criteria for materials were that they were documented as effective for one or more of the targeted features, that they could be taken orally, and that humans could safely ingest them. The size of the literature review precludes discussion here.

Dosages for the mice were reformulated based on amounts commonly prescribed to humans. Firstly, values were adjusted for the smaller body size of the mice. Next, the dosages were increased by a factor of ten based on the higher gram-specific metabolic rate (and consequently faster utilization and turnover) of mice compared to humans (54). The AASUP was prepared in liquid form, soaked onto a small piece of bagel and allowed to dry (dry weight of supplement = 140.3 mg per mouse based on a 35 g mouse). All treated mice received this dose midway through the photoperiod. The bagel bits were avidly eaten, ensuring mice obtained full and equivalent doses. Details of the ingredients are provided in Table I. This formulation was maintained for the duration of the study.

Results

Learning was assessed using two indicators; the number of trials required to learn the task (Fig. 1), and the total number of errors committed during learning (Fig. 2). In mice < 270 days of age (log 5.6, Fig. 1), untreated TGM (UTGM) made fewer errors and required fewer trials to learn than normals (Fig. 2). As early as 150 days of age, however, the learning ability of UTGM began to deteriorate (log 5.2, Figs. 1 and 2). This deterioration progressively increased until approx. 330 days, when most UTGM were unable to learn the task (log 5.9, Figs. 1 and 2). The lowest values for the number of trials to learn and total errors committed occurred when UTGM were approx. 100 days old. These scores were significantly lower than similarly aged normal mice and either group of untreated older mice (log 4.8, Figs. 1 and 2).

Age-related patterns for trials to learn were best fitted by second-order polynomial regression. The overall pattern of age-related learning (i.e., parabolic) strongly suggests that mice younger than 85 days of age did not learn as well as youthful adults. Indeed, the only mice that failed to learn the task were either quite young, or considerably older (log 3.9, Fig. 1). There were too few mice younger than 85 days of age, however, to specifically test this statistically. For UTGM, the regression of number of trials to learn against age was highly significant ($P < 0.000079$, $r^2 = 0.48$, $n = 32$). No age-specific pattern of learning was statistically resolved for normal mice ($P > 0.13$, $r^2 = 0.14$, $n = 32$). TGM treated with the dietary supplement (AASUP TGM) had qualitatively and quantitatively different age-related patterns of learning than UTGM. Age-related patterns for trials to learn for AASUP TGM were best described by a linear regression (Fig. 1, $P < 0.00058$, $r^2 = 0.42$, $n = 24$). Whereas old UTGM showed rapid deterioration in learning, AASUP TGM learned in progressively fewer trials.

Table I. Formulation of a Dietary Supplement Designed to Reduce Oxidative Stress and Inflammation, Maintain Membrane and Mitochondrial Integrity, and Enhance Insulin Sensitivity

Vitamin B1 ^b	0.72 mg/day	Flax Seed oil ^h	21.6 mg/day
Vitamin B3 ^b	0.72 mg/day	Folic Acid ^b	0.01 mg/day
Vitamin B6 ^b	0.72 mg/day	Garlic ^b	21.6 mcg/day
Vitamin B12 ^b	0.72 mcg/day	Ginger ^h	7.2 mg/day
Vitamin C ^b	3.6 mg/day	Gingko Biloba ^h	1.44 mg/day
Vitamin D ^b	2.5 IU/day	Ginseng (Canadian) ^h	8.64 mg/day
Vitamin E ^b	1.44 IU/day	Green Tea Extracts ^f	7.2 mg/day
Acetyl L-Carnitine ^c	14.4 mg/day	L-Glutathione ^a	0.36 mg/day
Alpha-Lipoic Acid ^e	0.72 mg/day	Magnesium ^b	0.72 mg/day
ASA ^d	2.5 mg/day	Melatonin ^g	0.01 mg/day
Beta Carotene ^b	50.0 IU/day	N-Acetyl Cysteine ^e	7.2 mg/day
Bioflavonoids ^h	4.32 mg/day	Potassium ^b	0.36 mg/day
Chromium Picolinate ^f	1.44 mcg/day	Rutin ^h	0.72 mg/day
Cod Liver Oil ^b	5.04 IU/day	Selenium ^h	1.08 mcg/day
CoEnzyme Q10 ^h	0.44 mg/day	Zinc (chelated) ^b	0.14 mg/day
DHEA ^g	0.15 mg/day		

Vitamin brands: ^aCell Life; ^bJamieson vitamins; ^cJarrow Formulas; ^dLifebrand; ^eNatural Factors; ^fNaka; ^gPromatrix; ^hSwiss vitamins; ⁱVitamin Power Inc.

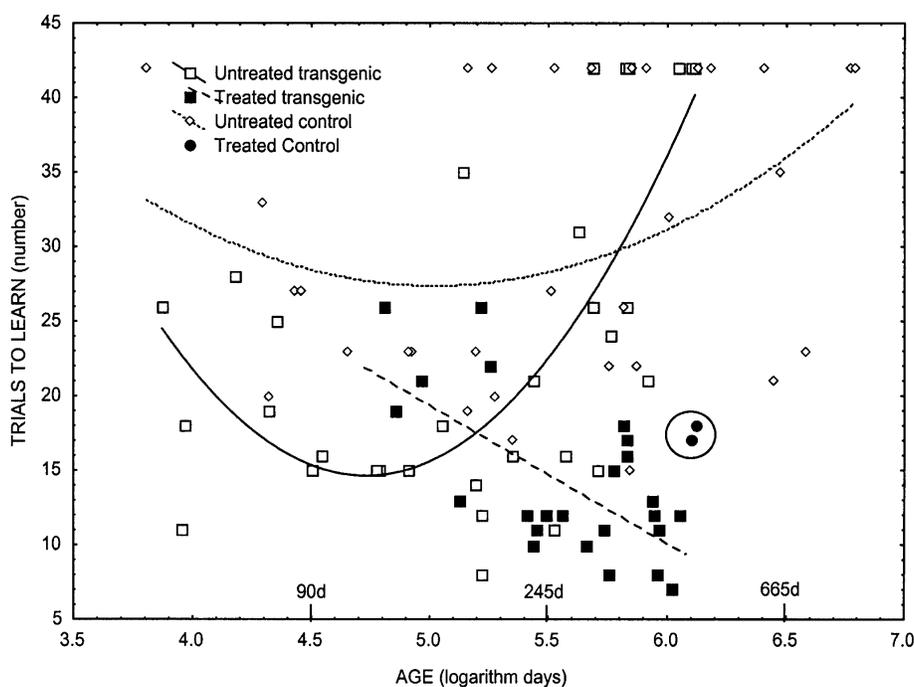


Figure 1. Number of trials required by TGM and age-matched normal controls to learn an eight-choice maze across lifespan. Regression lines had the following equations: untreated TGM ($y = 13.37(x^2) - 126.54(x) + 313.96$); untreated normal mice ($y = 3.72(x^2) - 36.92(x) + 118.76$) and treated (anti-aging supplement) TGM ($y = -9.19(x) + 65.49$). Age in days is indicated above the X axis.

Data for total errors were more variable than for trials to learn (compare Figs. 1 and 2); however, a second-order polynomial regression against age was again highly significant for UTGM ($P < 0.0016$, $r^2 = 0.36$, $n = 32$). As with trials to learn, normal untreated mice showed no significant age-related pattern ($P > 0.28$, $r^2 = 0.087$, $n = 32$). AASUP TGM showed linearly declining error rates with age ($P < 0.0043$, $r^2 = 0.31$, $n = 24$). For both trials to learn and errors committed, the age-related trends were in opposite directions for the two groups; a dramatic loss of cognitive function with increasing age in the UTGM and a progressive improvement in learning ability with age in AASUP TGM (Figs. 1 and 2).

To further test the effect of age on learning, untreated mice were assigned to two age classes (young < 270 days, $n = 20$ UTGM, 16 normals; and older > 270 days, $n = 12$ UTGM, 16 normals). To assess the effect of age and the AASUP on learning, and because of the unavailability of very young AASUP mice, AASUP TGM and UTGM were assigned to two age classes (younger > 120 days < 260 days, $n = 10$ UTGM, 11 AASUP TGM and older > 260 days, $n = 13$ UTGM, 13 AASUP TGM), based on the trends evident in the figures and the need to balance sample sizes and match age ranges. The focus of interest was the effects of the AASUP on older TGM and because of the substantial amount of time this cognition test takes (up to 7 days per

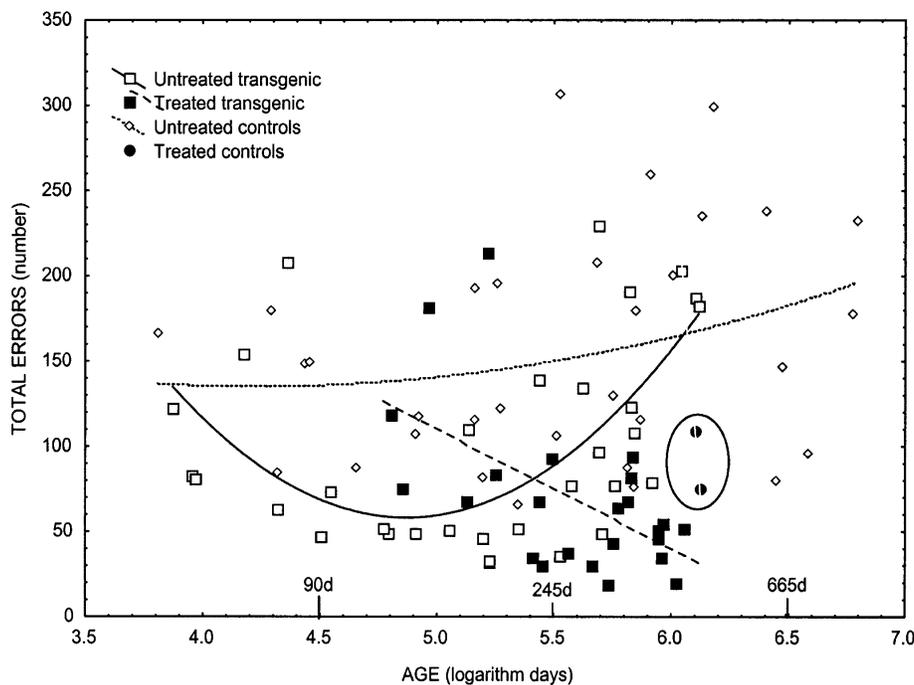


Figure 2. Total errors committed during learning an eight-choice maze by TGM and normal controls across lifespan. Regression lines had the following equations: untreated TGM ($y = 77.19(x^2) - 755.66(x) + 1888.15$); untreated normal mice ($y = 6.7(x^2) - 47.15(x) + 206.74$) and treated (anti-aging supplement) TGM ($y = -70.01x + 459.76$). Age in days is indicated above the X axis.

mouse) treated normal mice and very young AASUP TGM have not yet been tested. The data did not allow for a finer designation of age classes without loss of statistical power.

For untreated mice, both age and genotype of mouse were significantly different (ANOVA) for either trials to learn (Mouse type: $F = 20.46$, $df = 60$, $P < 0.00029$; Age: $F = 5.72$, $df = 60$, $P < 0.020$), or total errors (Mouse type: $F = 9.85$, $df = 60$, $P < 0.0026$; Age: $F = 10.27$, $df = 60$, $P < 0.0022$). There was also a significant interaction between age and genotype for trials to learn (i.e., learning changed with age differently between untreated normals and UTGM; $P < 0.029$). ANOVA for total errors yielded similar results except no significant interaction between age and genotype was resolved ($P > 0.36$). Newman-Keuls testing detected that young UTGM learned significantly better than older UTGM and either age class of normal mice ($P < 0.05$). Older UTGMs were not significantly different than normal mice, and no significant impact of age was detected in untreated normal mice.

Planned comparisons were performed with *t* tests. The average trials to learn for young UTGM was 17.71 ± 6.52 (mean \pm SD), about 43% less than older UTGM or either age class of normal mice (32.92 ± 10.17 , 28.13 ± 9.10 , and 33.25 ± 10.04 , respectively). Both age classes of AASUP TGM expressed significantly better performance than normal mice and older UTGM. Young AASUP TGM averaged 17.2 ± 6.3 (mean \pm SD), $n = 10$, trials to learn, a difference that was not statistically resolved ($P > 0.92$, $df = 18$) from young UTGM. The regression analysis, however, suggests a trend for reduced performance in young AASUP TGM (Fig. 1). The average number of trials to learn for older AASUP TGM was 11.8 ± 3.8 , $n = 14$, an improvement of about

260% ($P < 0.000013$, $df = 26$) over older UTGM. Remarkably, the performance of the older AASUP TGM was also about 30% better than either treated ($P < 0.016$, $df = 22$), or untreated younger TGM ($P < 0.035$, $df = 22$). The least number of trials to learn for each class of mice was: young UTGM (8 trials); older UTGM (15 trials); young untreated normals (15 trials); and older untreated normals (15 trials); young AASUP TGM (10 trials) and older AASUP TGM (7 trials).

The mean total errors for young UTGM was 77.85 ± 46.61 trials (mean \pm SD), whereas untreated young normals and older UTGM had higher and very similar scores (139.50 ± 60.30 and 138.50 ± 58.67 , respectively; i.e., young UTGM made about 44% fewer errors during learning). Older untreated normal mice committed the greatest mean total of errors at 172.69 ± 70.24 .

As for trials to learn, the number of errors committed by younger AASUP TGM (96.8 ± 59.7 errors) was not statistically resolved from young UTGM ($P > 0.12$, $df = 18$), despite an evident trend. Older AASUP TGM (50.7 ± 6.2 errors) learned significantly better than younger AASUP TGM ($P < 0.013$, $df = 22$), but older AASUP TGM did not differ from younger UTGM ($P > 0.35$, $df = 22$).

For total errors committed, older AASUP TGM had the best learning performance with a mean of 50.4 ± 21.4 errors, a substantial ($> 240\%$) improvement over older UTGM ($P < 0.00019$, $df = 26$). The least number of total errors committed for each class of mice was: older untreated normals (76 errors); older UTGM (49 errors); young untreated normals (47 errors); young UTGM (32 errors); young AASUP TGM (30 errors) and older AASUP TGM (19 errors). Because many older untreated mice did not learn the

task prior to the cut-off point (39 trials), their error rates and trials to learn are undoubtedly considerably underestimated (Figs. 1 and 2). Thus, a conservative estimate suggests that the learning of young adult TGM (treated or not) and older AASUP TGM was at least 200–250% better than untreated normal animals, or older UTGM.

Because many cellular signaling pathways require RONS, we considered that the AASUP might well have negative consequences on associated functions. Since mitogenesis is one such process, we analyzed the mice for possible growth impacts. Table II illustrates that there was virtually no impact of the AASUP on growth of either TGM or normal mice of either sex. TGM were larger than normal mice as expected. A surprising sexual dichotomy in body size expressed in TGM was also unaffected by the AASUP.

Two AASUP normal mice >540 days of age, learned in 17 and 18 trials while making 109 and 75 errors respectively (Figs. 1 and 2). The averages for these two mice (17.5 trials, 92.0 total errors) were much lower than those of age-matched untreated normal mice (average: 34.8 trials, 200.3 total errors, $n = 8$) or even young untreated normals (27.8 trials, 138.9 total errors, $n = 18$), suggesting that larger samples of very old mice may resolve positive effects of the AASUP on learning in normal animals.

Discussion

Results confirm the enhanced learning of this task by younger mature UTGM (18). A second-order polynomial regression best described age-related learning, identifying both rapid early declines in performance (Figs. 1 and 2) and inferior acquisition by younger UTGM (30–85 days of age). The AASUP effectively offset age-related cognitive deterioration (Figs. 1 and 2). The performance of AASUP TGM actually improved, older mice learning in fewer trials than younger treated or untreated TGM. This is remarkable considering that young UTGM express approximately 2-fold better performance than normal mice of any age. Several mechanisms likely explain these results, given the known

properties of the GH axis and TGM. Earlier reviews discuss more general aspects (18, 55).

Development. IGF-I is elevated 3-fold in TGM (56). IGFs regulate brain development, with IGF-I transgenic mice achieving brains 50% larger than normal. Alterations include the hippocampus, a structure highlighted in spatial memory (57). Mice deficient in IGF hormones have reduced brain size and hypomyelination (58). IGF-I, receptors for GH, IGF-I, and IGF-II, and IGF binding protein (IGFBP)-4 are strongly expressed in the hippocampus (2, 59) and may enhance memory (55).

IGF-I induces neurogenesis from progenitor cells in the dentate granule cell layer of the hippocampus (60), and adult neurogenesis contributes to formation of trace memories (61). IGF-I gene disruption reduces the granule cell layer of the dentate gyrus (58), whereas IGF-I overexpression increases cell numbers by 29–61% (57). IGF-I mRNA increases after brain injury, suggesting a regenerative or protective function (2). The only known genetic marker associated with exceptional learning in children is IGF-II (59).

Elevation of plasma IGF-I only occurs 2 weeks post-natally in TGM. Despite this elevation in IGF-I, the TGM brain was reported to be no larger than controls and decreased relative to body size as the mice grew (56). It remains that IGF-I increases neuronal complement and myelination and extends the post-natal period of brain maturation. The increased brain size of IGF-I transgenic mice occurs after birth (57). The immaturity of the hippocampus at birth may explain the poorer performance of very young mice (Figs. 1 and 2). The anatomy and micro-structure of the TGM brain warrants detailed examination, particularly the hippocampus (57, 60).

Apoptosis. GH and IGF-I are expressed in brain and also enter via regulated uptake mechanisms and cerebral spinal fluid (55). Elevated GH and IGF-I could increase youthful neuronal complement and offset losses in old age by inhibiting apoptosis (2, 57). Furthermore, chronic GH overexpression in TGM does not decline with age (62), unlike normal mammals. Alternatively, increasing GH-associated RONS (31) might override anti-apoptotic thresholds, exacerbating apoptosis, necrosis and neuronal excitotoxicity. Lymphocytes from UTGM are more sensitive to radiation-induced apoptosis than controls, implying that elevating free radicals may accentuate apoptosis if basal levels are already high. Lymphocytes from AASUP TGM and AASUP normal mice are resistant to radiation-induced apoptosis (unpublished). Similar results may extend to neurons.

Neurotransmitters. Many GH axis elements (e.g., GH, IGF-I, IGF-II, SRIF, IGFBPs), and their receptors are widely expressed in brain, often co-localized or associated with particular neurotransmitter systems. At least seven IGFBPs regulate IGF-I receptor activity and IGF-I availability (2, 55). The GH axis may influence memory by modulating cholinergic transmission, particularly in the septal-

Table II. Mean Mature Mass (g) of TGM and Normal Mice Reared on a Standard Diet or on Standard Diet with an Anti-Aging Supplement

Gender	Diet	Mean \pm SE mass (g)	n	Differs from
TGM				
Male	Supplement	54.6 \pm 1.95	15	b, d–h
Female	Supplement	46.5 \pm 0.74	32	a, c, e–h
Male	Standard	56.5 \pm 0.55	111	b, d–h
Female	Standard	46.8 \pm 0.33	141	a, c, e–h
Normal				
Male	Supplement	31.2 \pm 0.87	32	a–d
Female	Supplement	30.1 \pm 0.72	36	a–d
Male	Standard	32.9 \pm 0.49	55	a–d
Female	Standard	30.1 \pm 2.36	112	a–d

Significant differences ($P < 0.05$) among groups were obtained via ANOVA and a Newman–Keuls test.

hippocampal tract (18, 63). Fragments of IGF-I or GH bind to glutamate (NMDA) or opioid receptors, respectively (63, 64), and SRIF can bind GABA receptors (65). IGF-I modulates L-type Ca^{2+} channels and impacts Na^{+} channels crucial to depolarization (15). IGF-I alters glutamate receptor expression, and increases neuronal sensitivity to glutamate (including excitotoxicity; Ref. 2). IGF-I can elevate GABA release in the cerebellum (15). In rats, antibodies against IGF-I impair acquisition of aversive learning and reduce locomotion in the open field (2). Gene arrays may reveal complex neurotransmitter alterations in TGM.

Despite controversy, sleep is strongly associated with memory consolidation (66). The GH axis regulates sleep and EEG patterns (20, 21). Protein synthesis required for long-term memory (67) is elevated in sleep (68). TGM sleep 3.4 h longer than normal (19, 21), which could upregulate systems relevant to long-term memory. The GH axis appears to regulate large-scale coordination of brain functioning (e.g., brain waves and sleep-wake cycles), with associated neurotransmitter impacts in diverse brain regions (15, 18, 55). Future studies must consider temporal cycles.

Dopamine is strongly connected to the GH axis (69), and is reduced in several brain regions of TGM in association with elevated serotonin in others (26). TGM were more active in an open field test, indicative of reduced emotionality (25, 26). Environmental enrichment improved spatial learning in rats and was associated with increased SRIF and SRIF mRNA in the cortex. More rapid habituation to novelty was also observed (70). Given linkages of dopamine and serotonin to emotionality, it is significant that GH has antidepressant effects, and that antidepressants evoke GH secretion (71). Normal mice moved more quickly in our arena and some errors may have been due to their greater emotionality.

Transduction Pathways and Oxidative Stress. LTP and learning require activation of MAPK/ERK (and perhaps PI3K) by neurotransmitters (67, 72–75). Receptors for IGF-1, GH, and insulin occur on neurons in regions associated with cognition and memory (15, 18, 55), and also activate MAPK-ERK and PI3K. Activation of MAPK/ERK by neurotransmitters may require transactivation by growth factor receptors (76). Activation of these pathways generates and requires RONS. Sources include NAD(P)H oxidase systems, arachidonic acid metabolites, cyclo-oxygenase systems, nitric oxide synthase, and mitochondria. NAD(P)H oxidase generates superoxide radical (and secondarily H_2O_2), that are particularly important in early MAPK-ERK and PI3K signaling (42, 74, 77, 78). Neuronal NAD(P)H oxidase is also involved in apoptosis (79, 80), whereas mice with dysfunctional NAD(P)H oxidase are resistant to free radical injury (81).

Antioxidants can inhibit MAPK/ERK and LTP (82, 83). Transgenic mice over-expressing cytoplasmic or extracellular superoxide dismutase have defective LTP and memory (36, 84). Thus, antioxidants could negatively impact cognition, even though they might ameliorate age-

related deterioration. Learning of younger AASUP TGM was not statistically different than younger UTGM, which is reassuring, but a trend is apparent that might be resolved with larger samples (Figs. 1 and 2). Younger AASUP TGM also had higher scores and more variability in both parameters tested, particularly for total errors.

If learning requires an optimal level of RONS, then the improving performance of AASUP TGM with age could reflect increases in RONS sufficient to offset damping by the AASUP. Regardless, younger AASUP TGM still expressed superior maze learning than normal untreated mice, suggesting that cognitive enhancement without associated damage is possible.

RONS enhance phosphorylation of tyrosine and threonine kinases in transduction networks (required for MAPK/ERK activation), while reversing or inhibiting the action of antagonistic phosphatases (83, 85). IGF-I acts similarly to RONS (2). Chronic overexpression of GH/IGF-I could bias redox status and thus potentiate protein kinase phosphorylation and enhance signal transduction relevant to neurotransmission and synaptic LTP. Additionally, induction of MAPK-ERK by growth factors and/or neurotransmitters could promote oxidative stress or contribute to senescent phenotypes by differential activation of redox-sensitive signaling cascades, transcription factors, and gene expression (86).

Moderate increases in RONS can frequently elicit super-normal functioning whereas higher levels may inhibit signal transduction and damage nucleic acids, proteins and lipids. Severe damage induces apoptosis or necrosis. This extends to the MAPK/ERK pathway that can be either neuroprotective or apoptotic (83, 87). RONS generally express an inverted “U”-shaped dose–response curve for physiological impacts that may underlie the age-related changes in learning expressed by UTGM (Figs. 1 and 2).

Maintaining GH axis function attenuated age-related memory deficits (but not sensorimotor deterioration) in normally aging rats (88). In our model, upregulation of the GH axis is associated with remarkably enhanced task performance but subsequent premature and rapid cognitive declines associated with increasing RONS stress (17, 31). Possibly, RONS damage accumulates above a threshold that exceeds effective defense, repair or replacement, and TGM express such features early in life.

Better task performance of young UTGM over normal controls (18) may reflect RONS enhancement of signal transduction in neurons. As RONS increase with age they may then become inhibitory, induce damage or initiate apoptosis. Chronic or frequent upregulation of RONS processes could accumulate damage, even if short-term impacts are positive or enhancing. GH and IGF-I suppress antioxidant enzymes in cell cultures, and generally, GH axis activity negatively correlates with antioxidant activity (89).

Inflammation. Inflammatory processes are intimately linked to cellular damage associated with RONS. Inflammation involves RONS generation by immunocytes

via NAD(P)H oxidase systems similar to those found in neurons. Oxidative bursts by immunocytes are upregulated by GH (90). Aging may involve low-level inflammatory processes (17) that can be ameliorated by NSAIDs and antioxidants included in the AASUP (Table I; Ref. 5).

Brain Energy Supply. Energy supply strongly modulates cognition, as evidenced by enhancement of learning and memory by glucose (3). Energetic constraints are implicated in age-related cognitive dysfunctions and neurodegeneration (91, 92). The GH axis, particularly IGF-I, is critical in the development and maintenance of brain vasculature crucial to glucose supply and respiratory exchanges (93) and GH, IGF-I and SRIF all regulate energy balance.

GH favors mobilization of fatty acids and reduced adiposity (93). TGM express strong insulin resistance, hyperinsulinemia, altered PI3K signaling, but normoglycemia (10, 40). Such alterations likely reflect the role of the GH axis in sleep-associated metabolism, when glucose is sequestered for the brain while peripheral tissues develop insulin resistance and preferentially metabolize fatty acids. The reduced physical activity, increased sleep (19), and altered brain EEG patterns (20) of TGM likely reflect alterations in global energy allocation. Impacts of insulin on learning and LTP highlight alterations in glucose metabolism, and reduced insulin sensitivity is associated with several neuropathologies (92). Significantly, sucrose supplements completely restored normal activity and sleep patterns in TGM (21), suggesting alterations in brain vascularization and/or metabolism.

Insulin Sensitivity. Dietary restricted rodents, dwarfs and other models of downregulated GH axis function express high insulin sensitivity and extended longevity (94). Alternatively, TGM, obesity, type 2 diabetes, acromegaly and in many cases, aging, display insulin resistance and hyperinsulinemia (10, 92). Insulin may play a major role in aging and several neuropathologies and chromium picolinate (Table I) increases insulin sensitivity (92, 95). Antioxidants also effectively reduce insulin resistance (see Ref. 17). Impairment of glucose metabolism or mitochondrial substrate supply can increase production of free radicals (e.g., Ref. 35), which could accelerate cellular damage and aging. Insulin resistance can also increase glycation, another mechanism implicated in general aging (96). If the AASUP reduces insulin resistance, we expect associated reductions in plasma insulin.

Cellular and Mitochondrial Membranes. Cellular membranes are crucially involved in transport of cellular nutrients, maintenance of ion channels and functioning of receptors for hormones, cytokines, and neurotransmitters. Aging is associated with increasing levels of lipid peroxidation, changes in fatty acid composition and reductions in membrane fluidity. Such alterations have major impacts on all of the above functions (97), and RONS strongly contribute to age-related membrane alterations. Neuronal membranes are additionally involved in electrical signaling and are particularly rich in unsaturated fatty acids. Antioxidants,

anti-inflammatory and fatty acid components of the supplement (Table I) were included to prevent LP, maintain membrane fluidity and supply unsaturated fatty acids.

Mitochondrial functioning is crucial in aging, and interventions can maintain mitochondrial integrity (27–29). Mitochondrial membranes are particularly important as they support respiration and generation of ATP. The main lipid constituent of the inner mitochondrial membrane, cardiolipin, is composed largely of unsaturated fatty acids and shows age-related alterations that impact mitochondrial functioning and free radical generation (95, 98). Unsaturated fatty acids are more susceptible to LP that can actually exacerbate further generation of RONS. We reasoned that antioxidants would offset this possibility. Others recently showed that indeed, L-carnitine increases generation of free radicals, but this is prevented by α -lipoic acid (Table I; Ref. 27).

Risks. Our AASUP may lower RONS processes to levels that maintain enhanced cognition while still preventing damage or apoptosis associated with neuronal excitotoxicity. These results suggest great promise for clinical applications, but there are potential risks. Youthful cognition, growth or reproduction could be inhibited, particularly in normal mammals. Inhibition of immunological RONS bursting could compromise abilities to kill pathogens or destroy aberrant cells. Mice with such deficiencies are prone to infections (86). It is promising that growth of TGM and normal mice is not altered by the AASUP (Table II), suggesting that associated RONS generation is sufficiently maintained in mitogenic pathways. It is also noteworthy that the AASUP also had no effect on a sexual size dichotomy expressed only in TGM.

Suppression of RONS can also inhibit apoptosis, possibly increasing the risk of tumors. Generally, any intervention that extends longevity by offsetting a limiting pathology will allow expression of those next most limiting at later ages. Our supplement extends longevity of TGM, but their liver pathology is not strongly ameliorated (unpublished results). Consequently, we cannot yet exclude the possibility of toxicity. Notably the learning ability of UTGM closely paralleled survivorship (see Ref. 31). AASUP TGM, however, show no decline in learning ability, even when approaching death. We may, however, be achieving differential organ-specific results.

Our AASUP was formulated several years ago, and because we were interested in lifetime consequences, was maintained despite reformulations based on further scientific progress. Most other studies have treated old animals for much shorter periods (weeks to a few months). Reformulations do not include DHEA. PBN (α -phenyl-N-t-butyl nitron), known to offset cognitive aging in rodents (46), was not included originally because of uncertainty regarding safety. A breakdown product of PBN, N-t-butyl hydroxylamine may be more potent and non-carcinogenic (49).

Conclusion. TGM provide a valuable model of enhanced RONS processes with features resembling aging.

There are very few models of both early cognitive enhancement and accelerated age-related declines. Moreover, the ability to modify these processes via a dietary supplement provides a model that promises insights into both normal cognition and its age-related deterioration.

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