Chronic [D-Ala²]-Growth Hormone–Releasing Hormone Administration Attenuates Age-Related Deficits in Spatial Memory

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The age-related decline in growth hormone is one of the most robust endocrine markers of biological aging and has been hypothesized to contribute to the physiological deficits observed in aged animals. However, there have been few studies of the impact of this hormonal decline on brain aging. In this study, the effect of long-term subcutaneous administration of [D-Ala²]-growth hormone-releasing hormone (GHRH) on one measure of brain function, memory, was investigated. Animals were injected daily with 2.3 μg of [D-Ala²]-GHRH or saline from 9 to 30 months of age, and the spatial learning and reference memory of animals were assessed by using the Morris water maze and compared with those of 6-month-old animals. Results indicated that spatial memory decreased with age and that chronic [D-Ala²]-GHRH prevented this age-related decrement (24% improvement in the annulus-40 time and 23% improvement in the number of platform crossings compared with saline treated, age-matched controls; p < .05 each). No changes were noted in sensorimotor performance. [D-Ala²]-GHRH attenuated the age-related decline in plasma concentrations of insulin-like growth factor-1 (IGF-1) (p < .05). These data suggest that growth hormone and IGF-1 have important effects on brain function, that the decline in growth hormone and IGF-1 with age contributes to impairments in reference memory, and that these changes can be reversed by the chronic administration of GHRH.
chronic \[D\text{-Ala}^{2}\]-GHRH injections on several measures that are altered with age, including cognitive function, IGF-1 levels, body weight, survival, and pathology, were evaluated.

MATERIALS AND METHODS

Animals
Brown Norway \( \times \) Fischer 344 (F1) male rats were obtained from the National Institute on Aging colony at Harlan Industries (Indianapolis, IN) at 9 months of age and were housed in a specific pathogen-free facility until 30 months of age. These facilities are accredited by the American Association for Accreditation of Laboratory Animal Care. Rats were maintained on a 12-hour light, 12-hour dark cycle with food and water available ad libitum. Eighteen animals were randomly selected to receive daily subcutaneous injections of either normal saline (control) or 2.3 \( \mu \)g/200 \( \mu \)l of \[D\text{-Ala}^{2}\]-GHRH (Protein Synthesis Lab, Wake Forest University, Winston-Salem, NC) from 9 months to 31 months of age; 73% of the saline-treated and 72% of \[D\text{-Ala}^{2}\]-GHRH-treated animals survived through 28 months of age (the end of behavioral testing). The remainder of the animals died of normal age-related pathologies. One month before the initiation of behavioral testing, an additional cohort of young animals was obtained at 5 months of age and injected daily with saline for 1 month. Six young (6 month), six old control (28 month), and six old GHRH (28 month) animals were used for behavioral testing. An additional cohort of young animals was observed for 1 month. Six young (6 month), six old control (28 month), and six old GHRH (28 month) animals were used for behavioral testing. Injections were continued in each animal throughout all studies. Blood samples were taken from each animal under ketamine–xylazine anesthesia. Animals that died spontaneously during the course of the study were necropsied for gross pathologies by Animal Resources Personnel of the Wake Forest University School of Medicine.

Morris Water Maze

Training.—The water maze was a black plastic tank (MacCourt Products, Denver, CO), 1.67 m in diameter and 0.76 m in height. The tank was filled with water (24 ± 2°C) to a depth of 30 cm and made opaque through the addition of nontoxic, white powder tempera (Palmer Paint Products, Troy, MI). The escape platform (Plexiglas, 10 cm \( \times \) 10 cm) was 1 cm below the surface of the water. Animals were trained to locate and climb on the escape platform by using the straight swim method (28). A Plexiglas alley, 1.3 m long, 15 cm in width, and 60 cm in height, was placed in the tank and the escape platform was located at the end of the alley. Each rat was given one session of six trials, lasting until the animal climbed on the platform or for a maximum of 60 seconds. If the rat did not find the platform within 60 seconds, the animal was placed on the escape platform for 10 seconds. Swim time, the time to reach the platform, was recorded. Body temperature was maintained between trials by using heat lamps, and animals were towel dried. The time frame for all behavioral testing is indicated in Figure 1.

Place discrimination.—The place discrimination task distinguishes spatial reference memory in the water maze. The tank was divided into four quadrants, with the escape platform located in the NW quadrant, 25 cm from the edge of the tank. A start location in one of the three quadrants without the escape platform was determined randomly at the beginning of each session, and the start location was changed for each session while the escape platform remained in the same location throughout all four sessions. Two sessions were conducted per day with an intersession interval of 4–6 hours for a total of four sessions over 2 days. Each session consisted of six trials, five platform trials, and one probe trial, and each session was videotaped for subsequent analysis. Before the start of the first trial of the first session, the rat was placed on the escape platform for 10 seconds in order to permit orientation of the animal to the distal visual cues in the room surrounding the tank. The trial ended when the rat reached the platform (maximum swim time of 60 seconds). After completion of each trial, the rat was permitted to remain on the escape platform for 10 seconds and then returned to a holding cage. The rats were warmed with heat lamps during the 5- to 7-minute intertrial intervals. For the sixth trial, the probe trial, the escape platform was removed and the rat was tested for 60 seconds.

During the five platform trials of each session, the swim time was monitored and recorded. Lower scores in the platform trials indicated a faster rate of learning. To assess the rate of learning, learning ratios were calculated by using the following equations (26): (latency of trial 1 – latency of trial 2)/(latency of trial 1) and (latency of trial 1 – latency of trial 5)/(latency of trial 1). Higher scores in the learning ratios indicated better performance. During the probe trials, the annulus-40 time (the time spent in a 40-cm annulus around where the platform was located) and platform crossings (the number of times the platform location was crossed) were obtained. Higher scores in these measures indicated a better performance. Because a repeated measures analysis showed no significant change within groups across sessions, data were averaged across sessions.

Figure 1. Time line for behavioral tests; each block represents 1 day.
Sensorimotor Tests

Several measures were used to assess muscle strength, coordination, and vision. These included hanging from an inclined screen, swim distance in the probe trial, ability to see the raised platform in the water maze, and locomotion.

Hanging from an inclined screen.—The rat was placed facing upward in one compartment of a 60° tilted 1-cm mesh screen, and the time taken for the animal to fall onto a 7.6-cm foam pad was recorded with a maximum latency of 30 minutes.

Swim distance.—The distance that the rat swam during the probe trial was measured. The greater the distance traveled in this 60-second trial, the better the performance in this measure.

Ability to see the raised platform.—At the end of the place discrimination paradigm, the hidden platform was raised 2 cm above the surface of the water and placed in an area of the tank previously unused; the visual cues were removed. Each animal was placed in the tank and the latency to find the platform was recorded for one trial.

Locomotor activity.—Locomotor activity was assessed in Plexiglas test chambers (42 cm X 42 cm X 30 cm). Animals were habituated to the chambers for 60 minutes on the day prior to testing. Testing consisted of two sessions over 2 consecutive days with a latency of 60 minutes. Locomotor activity was measured by electronic counters that detected interruptions of eight independent photocell beams (Omnitech, Columbus, OH). Photocell counts were recorded at 5-minute intervals. The following measures were calculated: horizontal activity (total number of beam interruptions), forward locomotion [horizontal activity - (rearing + stereotypy)], vertical activity (rearing), and stereotypy (grooming).

Radioimmunoassay

Plasma levels of IGF-1 were measured by radioimmunoassay as previously described (29). IGF-1 (Bachem, Torrance, CA) was radiolabeled by using the lactoperoxidase, glucose oxidase method and purified on a Sep-Pak cartridge (Waters, Milford, MA). Plasma IGF-1 was extracted in acid-ethanol (30) and analyzed by radioimmunoassay (RIA), using antiserum obtained from National Institute of Diabetes and Digestive and Kidney Diseases and Dr. A. F. Parlow. Data are expressed in relation to the IGF-1 standard. The minimum detectable dose of this assay was calculated to be 15 pg/tube (90% B/B0), and 50% inhibition of tracer binding was 240 pg/tube. Serial dilutions of plasma extracted in acid-ethanol were parallel to the standard curve. The intra-assay and interassay coefficients of variance were 8% and 12%, respectively.

Statistical Analysis

Data for each study were analyzed using SYSTAT 8.01 (SPSS, Chicago, IL). Dependent variables measured in these animals included annulus-40 time, platform crossings, learning ratios, plasma IGF-1 levels, swim distance, and locomotor data. For the overall experiment error rate to be controlled, data were initially analyzed with a multivariate analysis of variance (MANOVA). Because this analysis indicated statistical significance [Wilks lambda = 0.327, F(16,124) = 7.99, p < .001], univariate ANOVAs were performed with a one-way (group) design. The latency to platform was analyzed by repeated measures ANOVA with a within-subjects design. The initial analysis indicated no Group X Session interaction F(6,60) = 0.866, p = .525. Therefore, data were subsequently analyzed with a Group X Trial design. The student Neuman Keuls post hoc test was used to make comparisons between groups when these analyses revealed significant significance (p < .05).

RESULTS

Straight Swim

The latency to reach the platform declined from trial 1 to trial 5 of the straight swim in the young, old saline-treated, and old GHRH-treated animals, indicating an improvement in performance [main effect of trial: F(4,60) = 10.24, p < .001]. Although a main effect for group, F(2,15) = 25.58, p < .001, was observed (differences between young and either old saline-treated or GHRH-treated animals, p < .05), no Group X Trial effects F(8,60) = 1.63, p = .14, were evident. The mean latency for trial 5 of the straight swim was 4.11 ± 0.31 seconds for young, 5.97 ± 1.24 seconds for old saline-treated, and 7.02 ± 1.60 seconds for old GHRH-treated animals (p > .1).

Spatial Reference Memory

Spatial reference memory declines in the aged animal, and chronic [D-Ala²]-GHRH administration attenuates this decline. An analysis of the learning curves (derived from the latency to reach the platform over trials) indicated significant differences among the three groups [main effect of group: F(2,15) = 4.82, p = .038]. Specifically, young animals exhibited shorter latencies to platform than old control or old GHRH-treated animals (p < .01). In addition, a significant decline in latency across trials [main effect of trial: F(4,60) = 2.55, p < .05] was evident (Figure 2).

![Figure 2. Latency to platform in the Morris water maze: a comparison of learning curves for young, old saline-treated and old GHRH-treated animals. The asterisk denotes p < 0.05, comparing young with old saline-treated or old GHRH-treated animals. Data represent the means ± SEM for six animals/group.](https://academic.oup.com/biomedgerontology/article-abstract/55/2/B106/570759)
The mean swim time for trial 5 was 5.20 ± 0.70 seconds, 20.68 ± 3.92 seconds, and 19.65 ± 4.50 seconds in young, old saline-treated, and old GHRH-treated animals, respectively. Nevertheless, an analysis of the Group X Trial interaction failed to reveal a significant difference; F(8,60) = 0.64, p > .05. An assessment of the rate of learning by an analysis of the learning ratios indicated a trend toward a decline with age (p < .08), but no statistical differences were observed among the treatment groups (p > .5; data not shown).

An analysis of the probe trial indicated overall differences between the number of platform crossings, F(2,15) = 10.10, p < .002, and annulus-40 time, F(2,15) = 17.26, p < .001. Specifically, a 48% decline in the annulus-40 time (p < .001) and a 45% decline in the number of platform crossings (p = .011) during the probe trial was observed in the aged control animals as compared with young control animals (Figure 3). These deficiencies represent impairments in the spatial reference memory of aged animals.

Aged animals that received injections of [D-Ala²]-GHRH demonstrated a profound reversal of the memory loss that occurs with age. This reversal was demonstrated by a 24% improvement in annulus-40 time (p = .007) and a 23% improvement in platform crossings (p = .032) during the probe trial for aged animals compared with saline-injected controls. In addition, measures of reference memory in the aged animals treated with the GHRH analog were not significantly different from those of young animals (Figure 3).

**Chronic [D-Ala²]-GHRH Does Not Alter Sensorimotor Measures**

An analysis indicated overall significance for ambulatory activity [F(2,27) = 6.136, p = .006], rearing activity [F(2,27) = 16.602, p < .001], forward activity [F(2,27) = 12.15, p < .001], and swim distance [F(2,15) = 4.68, p = .026]. In each of these measures, there were significant differences between young and old animals, regardless of treatment with saline or [D-Ala²]-GHRH (Table 1). The absence of differences in swim distance, locomotor activity, hanging from an inclined screen, and vision (data not shown) between old saline-treated and old GHRH-treated animals suggests that the improvements in the probe trial of the Morris water maze had a direct effect on spatial memory (Figure 4).

**[D-Ala²]-GHRH Attenuates the Age-Related Decline in IGF-1**

An analysis indicated overall differences of plasma IGF-1 with age and treatment, F(2,15) = 11.64, p < .001. Because many of the effects of growth hormone are mediated by IGF-1, it is possible that the cognitive effects of [D-Ala²]-GHRH in the aged rat result from an upregulation of IGF-1. In this study, chronic [D-Ala²]-GHRH administration resulted in a significant rise in growth hormone levels within 10 minutes of injection (data not shown). A significant decline in plasma IGF-1 was found with age when young and old vehicle-treated animals (p < .05) were compared, and chronic [D-Ala²]-GHRH administration partially attenuated the decline in plasma IGF-1 in a comparison with old control animals (p < .05; Table 2), suggesting that circulating IGF-1 may have an important role in regulating the age-related decline in brain function.

**DISCUSSION**

It is well documented that learning and memory decline with age (31–33). This age-related decline in memory has been reported with a variety of experimental paradigms and in every species studied to date, and it indicates that aged rats acquire accurate spatial memory at a slower rate than younger animals. These age-related cognitive deficits are observed in both reference memory tasks, such as the Morris water maze and the Barnes maze (32,34,35), and in tasks that emphasize working memory, such as the radial arm maze and alternation tasks (36,37). Similarly, delayed-nonmatching-to-sample tasks in nonhuman primates, which resemble clinical tests that assess memory function in humans with neurological disorders (38),...
support the conclusion that cognitive impairments are evident with age (39-41). Furthermore, many studies indicate that humans also undergo memory impairments with age (42,43). Although much research has been undertaken to understand the mechanisms of learning and memory and the etiology of the decline in these processes with age, the specific mechanisms for these impairments remain elusive. In the present study, we have hypothesized that cognitive changes with age result, at least in part, from age-related deficiencies in growth hormone and IGF-1.

As expected, our studies indicate that chronic [D-Ala²]-GHRH administration increased growth hormone pulse amplitude and partially attenuated the age-related decline in plasma IGF-1. More importantly, however, our data indicate that chronic [D-Ala²]-GHRH injections prevent age-related memory impairments that are manifest in aged rodents. Both the annulus-40 time and the number of platform crossings (measures of spatial reference memory) improved by 23-24% in old GHRH-treated animals compared with vehicle-injected animals. Furthermore, data indicated that the rate of learning declined with age as has previously been reported, but we did not find an effect of [D-Ala²]-GHRH on learning. Although the specific mechanisms for this disparity between learning and memory are unclear, it has been reported that these processes are not completely independent. Although learning must precede memory, the rate of learning is not always indicative of strength of memory acquisition. The data presented in this study are consistent with other treatments that improve spatial memory (including nerve growth factor and IGF-1) but do not influence learning (26,44,45).

Because many age-related physiological changes are reversed or prevented by replacement of either growth hormone or IGF-1 and because other studies demonstrate that acute GHRH administration does not have an effect on memory (46), we hypothesize that the effects of [D-Ala²]-GHRH are mediated through growth hormone and/or IGF-1 and that maintaining plasma levels of these hormones throughout life ameliorated the age-related decline in spatial memory. Certainly, further research into the mechanisms of action of GHRH on memory process will be necessary.

Although the specific mechanisms of action of GHRH in enhancing cognitive function are unknown, related studies suggest that these effects may be mediated by means of IGF-1. Plasma IGF-1 has been reported to be transported across the blood–brain barrier, and brain levels of IGF-1 decrease by 36% with age (47). In addition the administration of growth hormone to old animals has been reported to increase cortical levels of IGF-1 (48). When aged animals are infused with intracerebroventricular (icv) IGF-1, improvements in cognitive ability are also observed. Markowska and colleagues (26) demonstrated that 28 days of icv IGF-1 reversed the age-related decline in both spatial reference and working memory. Although the mechanisms whereby IGF-1 attenuates memory impairments are currently unknown, preliminary data from our laboratory suggest that IGF-1 may reverse the age-related decline in specific NMDA receptor subtypes and increase the activity of D₃–receptors, both of which are implicated in learning and memory (49–52). In addition, previous studies have suggested that IGF-1 stimulates several neurological processes, including differentiation of cortical neurons (53,54), oligodendrocyte proliferation (55,56), neurite outgrowth (57), and acetylcholine synthesis and release (58), and that it protects hippocampal neurons against β-amyloid-induced toxicity (59). These studies indicate that the age-related decline in cortical and plasma IGF-1 may result in cognitive deficits associated with age and that attenuation of the decline in IGF-1 may reverse these impairments.

Recent studies also suggest that a close interaction exists between angiogenesis and behavioral performance (60–62). Previous studies indicate that angiogenesis is necessary for...
synaptogenesis (63) and that age-related deficits in neuronal plasticity occur, at least in part, from an inability to generate new cerebral microvessels (64). One prominent effect of aging is a rarefaction of cerebral vasculature, and recent data suggest that growth hormone administration increases cerebrovascular density in aged animals (25). In addition, the cerebrovasculature is a source of several growth factors, including IGF-1 (65) and nerve growth factor, which are known to have an important role in memory (66–68), and it has been hypothesized that IGF-1 derived from vessels may provide trophic support for surrounding tissues. In previous studies, a rarefaction of total vessel number was hypothesized to be a contributing factor in the decline of IGF-1 protein levels in the cortex (47). However, the regulation of IGF-1 production by vessels and the age-related changes in vessel response to IGF-1 have not been assessed. Thus, the concept that growth hormone and IGF-1 influence vasculature rarefaction of cerebral vasculature, and recent data suggest that cerebral microvessels (64). One prominent effect of aging is a plasticity to occur, at least in part, from an inability to generate new tissue. In previous studies, a rarefaction of total vessel number was hypothesized to be a contributing factor in the decline of IGF-1 protein levels in the cortex (47). However, the regulation of IGF-1 production by vessels and the age-related changes in vessel response to IGF-1 have not been assessed. Thus, the concept that growth hormone and IGF-1 influence vasculature rarefaction with age is well supported by recent experimental data, whereas the effects of these hormones on brain blood flow and vascular regulated trophic support require further study.

The effects of GHRH on memory in the present study do not appear to be mediated by increased lifespan or alterations in the incidence of age-related pathologies. An analysis of a larger cohort of animals indicated that there were no changes in survival to 30 months of age, and necropsies conducted at the end of the study suggested no alterations in the incidence of disease normally associated with aging. Similarly, differences between the aged animals were not due to visual acuity changes, because independent tests confirmed that all animals could locate the platform when it was placed in a raised position. Finally, sensorimotor measures did not indicate a difference between the aged, GHRH-treated animals compared with age matched, saline-treated animals. This latter effect was surprising because growth hormone and IGF-1 have a number of actions on peripheral tissues, and it suggests that the modest increase in growth hormone and IGF-1 observed in this study with the chronic administration of [D-Ala²]-GHRH has effects on the brain, whereas higher doses may be necessary for peripheral effects. It is theoretically possible that minor improvements in sensorimotor performance (beyond that detectable in this study) contributed to the effects of GHRH on cognitive performance. In the case of ambulatory activity, for example, GHRH induced a 10.6% increase in activity, whereas a 19.3% increase (at 80% power) would be necessary to detect a significant increase at the 0.05 level. At the present time, it is unknown whether small increases in ambulatory activity of the order found in our results have the ability to influence cognitive performance in the Morris maze. However, even an increase of nearly 20% would not substantially account for the improvements in cognitive function, especially as other locomotor activities (e.g., swim distance and rearing) were actually decreased by GHRH treatment (compared with saline-treated animals). Therefore, we conclude that these data support the concept that GHRH has the ability to specifically prevent the decline in spatial reference memory with age.

CONCLUSIONS

In conclusion, our studies suggest that activity of the growth hormone/IGF-1 axis is essential for normal cognitive function and that deficiencies in this pathway contribute to the memory deficits associated with age. In this report, we find that plasma IGF-1 is maintained in the aged animal with daily injections of GHRH, and we propose that GHRH acts, in part, through the growth hormone/IGF-1 axis. Additional studies will be necessary to validate this hypothesis. Nevertheless, with the advent of orally active growth hormone–releasing factor analogs, the potential may exist to prevent or delay the onset of memory deficits that are present in many elderly people.

ACKNOWLEDGMENTS

We thank Sean A. Bennett, Paula T. Cooney, Arri S. Khan, and Colleen D. Lynch for excellent technical assistance. This research was supported by grant PO1 AG11370 from the National Institute on Aging. Phillip L. Thornton was supported by a predoctoral fellowship from the PhRMA Foundation. Materials for the analysis of IGF-1 were the generous gift of Dr. A. Parlow and the National Hormone and Pituitary Program and National Institute of Diabetes and Digestive and Kidney Diseases.

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