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Region specific neuron loss in the aged canine hippocampus is reduced by enrichment

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Abstract

Neuron loss within the hippocampus and entorhinal cortex occurs as a function of age in humans. We first tested the hypothesis that neuron loss occurs in the aged dog. The total unilateral number of neurons in the canine entorhinal cortex and subdivisions of the hippocampus from the left hemisphere were estimated using the optical fractionator. The brains from 5 old (13.0 – 15.0 years old) and 5 young (3.4 – 4.5 years old) beagle dogs were analyzed. The hilus of the hippocampus showed a significant loss of neurons (~30%) in the aged dog brain compared to young. Differences were not detected in the remaining hippocampal subfields and entorhinal cortex. We further tested the hypothesis that an antioxidant fortified food or behavioral enrichment would reduce the age-related loss of hilar neurons. Behaviorally enriched aged dogs had more neurons in the hilus (~18%) compared to aged controls. These results suggest that the aged canine hippocampus in the left hemisphere shows selective neuron loss and that behavioral enrichment may reduce this loss.

Keywords

dog; brain; aging; hippocampus; neuron loss; antioxidants; enrichment

1. Introduction

The hippocampus and entorhinal cortex are brain regions essential for intact cognitive abilities and appear to be particularly vulnerable to the aging process. In normal human aging, stereological studies have revealed neuron loss in the hilus and subiculum of the hippocampus (70,89,92), and within islands in layer II of the entorhinal cortex (37,69). Neuron loss also occurs in the hilus of the hippocampus of aged rats (7,11,67), but not in any area or layer of

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Disclosure Statement

The authors have no conflicts of interest.

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the entorhinal cortex, even when cognitive status is considered (45). By contrast, neuron loss has not been reported in any subregion of the hippocampus (41) or any layer of the entorhinal cortex of non-human primates (25,46).

Neuron number and function may be modulated by environmental enrichment. Environmental enrichment involves rearing animals in a socially and physically stimulus-rich environment (86). This typically involves housing animals in groups to increase social contact, and adding novel objects to the immediate environment to stimulate exploratory behavior. Environmental enrichment in mice increases the number of hippocampal dentate gyrus neurons and neurogenesis (40).

Neuron number and function may also be compromised by oxidative damage. Increasing oxidative damage to proteins, lipids and nucleotides may contribute to neuron dysfunction in normal and pathological aging in humans (3,31). Mitochondria are a source of damaging free radicals and they are in turn, particularly vulnerable to free radicals. Oxidative damage produces mutations in mitochondrial DNA, alters membrane fluidity and phospholipid composition, and leads to dysfunctional mitochondrial proteins (68). The result is a loss of biochemical and physiological function of mitochondria in the cell, which impairs normal cellular activities. In support of the hypothesis that oxidative damage may lead to neuron death, providing rats with Vitamin E increases neurogenesis (12,15,18) and improves cell survival in the dentate gyrus (14,18,23).

The aging dog exhibits many features of normal brain aging that develop in humans. Aged dogs show declines in cognitive function (36,47,81,82,83,85), decreased brain volume (77, 78,80,84), behavioral alterations (33,72,74,75), and neuropathology including the accumulation of human-type beta-amyloid (34,66,95), increased oxidative damage (35,56, 64,76) and apoptosis (4,42). Previous studies that examined neuron number in the canine brain report significant decreases with age in the cingulate gyrus, superior colliculus and claustrum (8,53). Calbindin-positive GABAergic neurons in the prefrontal cortex of the canine brain are also vulnerable to aging (61). No evidence of neuron loss, however, has been reported in other brain regions including the hippocampus in the aged dog.

The goal of the present study was to examine the effect of age on neuron number in the canine hippocampus and entorhinal cortex using the optical fractionator in the left hemisphere. We hypothesized that neuron loss in the canine hippocampus, like that in humans, will be region specific and may also occur in the entorhinal cortex. We further tested the hypothesis that an antioxidant fortified food or behavioral enrichment or both would reduce age-related loss of neurons or increase the number of neurons relative to untreated control animals. We further examined the relationship between neuron number and cognitive function to determine whether changes in neuron number could account for the cognitive enhancing effects of the behavioral enrichment and antioxidant fortified food.

2. Materials and Methods

2.1 Aging study animals

The brains from 5 aged (13.0 – 15.0 years old) and 5 young (3.4 – 4.5 years old) Beagles from the Lovelace Respiratory Research Institute (LRRI, Albuquerque, NM) breeding colony were used to examine age-related differences in neuron number in the hippocampus and entorhinal cortex. Unilateral neuron numbers were acquired for each subregion of the left hippocampus separately. Unilateral neuron numbers were determined for the left entorhinal cortex and in layer II specifically.

2.2 Treatment study animals

Twenty-four reproductively intact dogs aged 7.8–12.1 years at the start of the study were obtained from LRRRI and were divided into 4 treatment groups based on cognitive ability (48, 49, 50, 51). All dogs were evaluated at regular intervals over the duration of the study by a veterinarian and determined to be in good health. After 2.8 years of treatment, tissue was available for 6 dogs that received the diet, 5 dogs that received the behavioral enrichment, 6 dogs receiving a combined treatment and 5 dogs that did not receive either treatment. At that time the dogs ranged in age from 10.7–15 years of age. The 5 dogs that did not receive either treatment were also the aged dogs used for the age comparisons. The treatment study animals were cognitively assessed over the course of the study, which provided behavioral correlates to assess the functional significance of neuron numbers. Animals in the treatment study have been described in several publications (48, 49, 50, 51, 73) as part of a longitudinal study of aging and the effects of chronic treatment with behavioral enrichment and/or an antioxidant fortified food.

2.3 Dietary intervention

The control and antioxidant fortified foods were formulated to meet the nutrient profile for the American Association of Feed Control Officials recommendations for adult dogs (5). Control and test foods were identical in composition, other than inclusion of a broad-based antioxidant and mitochondrial cofactor supplementation to the test food. The control and fortified foods had the following differences in formulation on an as fed basis respectively: dl-alpha-tocopherol acetate, (120 ppm vs approximately 1000 ppm), l-carnitine (< 20 ppm vs 250–300 ppm), dl-alpha-lipoic acid (<20 ppm vs approximately 120 ppm), ascorbic acid as Stay-C (< 30 ppm vs approximately 80–100 ppm), and 1% inclusions of each of the following (1 to 1 exchange for corn): spinach flakes, tomato pomace, grape pomace, carrot granules and citrus pulp. The food was produced by an extrusion process and was fed for no more than 6 months before a new lot was milled.

2.4 Behavioral enrichment

The behavioral enrichment protocol consisted of social enrichment, by housing animals in pairs, environmental enrichment, by providing play toys on a weekly rotation, physical enrichment, by providing two 20-minute outdoor walks per week, and cognitive enrichment, through continuous cognitive testing. The cognitive enrichment consisted of a landmark discrimination task within 2 weeks of starting treatment (47, 48), an oddity discrimination task after 6 months of treatment (51), and size concept learning after 1.5 years of treatment (73, 85).

All animals, regardless of treatment group were evaluated on a test of spatial memory annually; at baseline, 1 and 2 years after the start of treatment (13), size discrimination and reversal learning at the 1 year timepoint (49, 50, 82), and black/white discrimination and reversal at the 2 year timepoint (24, 49). Dogs were euthanized at the end of the black/white discrimination and reversal learning task.

2.5 Tissue preparation

While under anesthesia (5% isoflurane), dogs were ex-sanguinated by cardiac puncture and within 15 minutes the brain was removed from the skull. The brain was sectioned midsagittally, with the entire left hemisphere being immediately placed in 4% paraformaldehyde for 48–72 hours at 4°C then transferred to phosphate buffered saline with 0.05% sodium azide at 4°C for long term storage. The left hemisphere was sent to NeuroScience Associates for sectioning. NeuroScience Associates treated individual canine hemispheres with 20% glycerol and 2% dimethylsulfoxide to prevent freeze-artifacts and subsequently embedded two hemispheres

(i.e. two animals) per block in a gelatin matrix using MultiBrain Technology™ (NeuroScience Associates, Knoxville, TN; www.neuroscienceassociates.com/multibrain.htm). After curing in formaldehyde, the block of embedded hemispheres was rapidly frozen by immersion in isopentane chilled to -70°C with crushed dry ice and mounted on a freezing stage of an AO 860 sliding microtome. The MultiBrain™ block was sectioned coronally at $40\mu\text{m}$. All sections cut (none were discarded) were collected sequentially into a 4×6 array of containers which were filled with Antigen Preserve solution (50% PBS pH 7.0, 50% Ethylene glycol, 1% Polyvinyl Pyrrolidone) for sections to be immunohistochemically stained. At the completion of sectioning, each container held a serial set of one-of-every-24th section (or one section every $960\mu\text{m}$). A total of approximately 1400 sections was generated per hemisphere. One container of free-floating sections was randomly selected per dog and all sections in that container that included the hippocampus or entorhinal cortex were collected for immunohistochemical staining.

2.6 Immunohistochemistry

Standard immunohistochemical methods were used and have been published elsewhere (17, 34). Briefly, neurons were identified using anti-mouse-neuronal nuclei (NeuN) at 1:1100 (Chemicon International, Temecula CA). Bound secondary biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) antibody was detected using ABC peroxidase kits and NeuN immunostaining was visualized using a SG Blue substrate kit, both from Vector Laboratories (Burlingame, CA). Free-floating sections were mounted onto gelatin coated slides, dehydrated through ethanols and coverslipped with DePeX mounting medium. Control experiments where primary or secondary antibody was omitted resulted in negative staining. One section from each dog was stained simultaneously in one immunohistochemistry run to maintain consistent reagent and dilution parameters and to reduce variability. NeuN was chosen because atrophic neurons can be confused with glia when Nissl stains are used (43) and NeuN can be used to confirm the identity of small cells in Nissl staining (7,26). To verify that the antibody penetrated the full thickness of the section, we used a systematic uniform random sampling scheme to measure the thickness of the section at 508 places (approx 100 per group of dogs) from all sections sampled from each animal used in the study using a 60x oil immersion lens. From each section at least 2 different sites were measured. Penetration of the antibody was complete since labeled cells were detectable in all deeper layers within a section. There were no significant differences in postprocessing tissue thickness between the groups of animals (young = $19.78\pm 0.87\mu\text{m}$; old 19.76 ± 1.04 ; antox 18.91 ± 0.99 ; enrich 19.00 ± 1.24 ; antox-enrich 18.94 ± 0.95).

2.7 Stereological evaluation

The optical fractionator technique (94) was used to estimate the total unilateral neuron number in all subregions of the hippocampus and the entorhinal cortex. Counting was performed using an Olympus BX50 microscope (Olympus, Tokyo, Japan) equipped with a motorized stage (Prior Scientific Inc., Rockland, MA) connected to a digital microcator (Heidenhain, Germany), and a video camera (JVC, Japan) connected to a computer with the CAST-GRID software package (Olympus, Copenhagen, Denmark).

All parameters for the canine hippocampus and entorhinal cortex were determined in a pilot study such that the coefficients of error for individual estimates were less than 10% (based on 29,41). The experimental parameters used are listed in Table 1. Estimates were based on counting NeuN positive nuclei as they came into focus.

Individual estimates of the total unilateral neuron number (N) for each region were calculated according to the following formula: $N = \Sigma Q^- \times 1/\text{ssf} \times 1/\text{asf} \times 1/\text{tsf}$ where ΣQ^- is the sum of counted neurons, ssf is the section sampling fraction, asf is the area sampling fraction, and tsf

is the thickness sampling fraction. The thickness of the section was used as the height of the disector making the tsf equal to 1. Optical fractionator rules typically require the use of guard zones at the upper and lower surfaces of the section. In the current study, we used a modified optical fractionator technique in which we did not use guard zones making the height of the counting frame equal to the section thickness. Several authors have used full section thickness and indicate that undercounting may occur (19,32,54). We used a systematic uniform random sampling scheme to measure the depth of 523 neurons (approx 100 per group of dogs) and found that the plots of cell depth looked similar between groups (Figure 1). These data indicate that this counting procedure may have yielded results that were an undercount of neurons because of the inability to compensate for lost caps or cells damaged at the inclusion surface (44). The top plane was used as the exclusion plane for the counting frame.

2.8 Delineation of the hippocampal subregions

The delineation of the hippocampal subregions was based on descriptions of human and rhesus monkey (30,41,89,91) and on canine hilus descriptions (10,39,65,87). Figure 2 shows the subregions of the canine hippocampus and the entorhinal cortex. All delineations were made using a 4x objective lens and subsequent counting performed using a 60x oil immersion lens for a final onscreen magnification of 2326x. The granule cell layer of the dentate gyrus forms a distinct cell layer of small and densely packed cells. The hilus was defined by its border with the granule cell layer and by lines drawn from the tips of the granule cell layer to the clearly distinguishable proximal tip of the CA3 pyramidal cell layer. The proximal area of CA3 (pCA3) began as a hook and continued to the point where the cells became more tightly packed, which is where the distal area CA3 (dCA3) began. The dCA3 region continued to the point where the packing of the cells decreased. From this point CA2 was defined until the packing of neurons decreased again and began to form the two layers of region CA1. CA1 continued to the border with the subiculum where neuron appearance and size changed. No attempt was made to divide the subiculum into subregions and thus our subiculum included the pre- and para-subicular regions.

The canine entorhinal cortex was identified, based on descriptions (2,96,97) as beginning after the subicular region and continuing to the rhinal sulcus (see Figure 2). Layer II was identified as the darker band of pyramidal neurons close to the outer surface of the entorhinal cortex.

2.9 Data analysis

A multivariate analysis of variance (MANOVA) was performed with age as the between subject variable and neuron numbers for each region as the dependent measures.

For the treatment study, a 2-way MANOVA was performed with food and enrichment as the between subject factors and neuron numbers for each region as the dependent measures. Pearson product correlations were used to examine the relationship between hilus neuron number and cognitive data collected during the treatment period. Bonferroni corrections for the multiple correlations required a significance level of $p < 0.0083$. All statistical analyses were performed using SPSS for windows version 13.0.

3. Results

3.1 Aging study

Tables 2 and 3 show a significant and selective age-related loss of neurons in the hilus of the hippocampus [$F(1,8) = 10.28, p = .012$]. There were no significant differences in neuron number between the young and aged dogs in the granule cell layer of the dentate gyrus ($p = .546$), area pCA3 ($p = .476$), area dCA3 ($p = .662$), total area CA3 ($p = .869$), area CA2 ($p = .809$), area CA1 ($p = .915$), the subiculum ($p = .280$) of the hippocampus, or within the entorhinal

cortex ($p = .561$) or layer II of the entorhinal cortex ($p = .584$). Islands in the entorhinal cortex as shown in Figure 2 were apparent but could not be clearly delineated on all sections for accurate counts, thus total layer II neuron estimates were used for analysis and served as a more conservative measure of possible entorhinal neuron loss.

3.2 Treatment study

Comparing the different treatment groups did not reveal significant differences in total neuron number in the hippocampus for either the enrichment condition ($p = .779$), or the antioxidant fortified food ($p = .362$). There were no differences in neuron number of the entorhinal cortex in the enrichment condition ($p = .378$) or the antioxidant fortified food ($p = .305$).

Because the hilus was vulnerable to aging, we compared neuron number in this subregion across the treatment groups. The behaviorally enriched dogs had significantly more neurons in the hilus of the hippocampus compared to aged-matched controls [$F(1,20) = 4.862$, $p = .041$] (Figure 3). Hilar neuron number was not completely restored to young animal levels as the behaviorally enriched aged dogs still had significantly fewer neurons compared to young dogs [$F(1,14) = 10.17$, $p = .007$]. This effect was selective for the enrichment as the antioxidant fortified food had no effect on neuron number in the hilus ($p = .584$).

3.3 Cognition

The aged dogs in the treatment study were tested on several cognitive tasks during the 3 years of the study prior to euthanasia. Correlations between hilar neuron number, which was sensitive to age and treatment, and the standard cognitive tasks used during the longitudinal study are listed in Table 4. There were no significant correlations after Bonferroni correction which required $p < .0083$.

4. Discussion

This study examined neuron number as a function of age in the canine hippocampus and entorhinal cortex of the left hemisphere using the optical fractionator. The present results reveal a significant subregion specific age-related loss of neurons in the hilus of the canine hippocampus that is similar to observations in normal aged humans. Further, hilar neuron number in aged animals could be modified by providing animals with a program of behavioral enrichment but not with an antioxidant fortified food.

4.1 Age-related neuron loss in humans and animals

We hypothesized that aged dogs would show a pattern of neuron loss in the hippocampus and entorhinal cortex similar to that observed in aging humans. A significant loss of neurons was found in the hilus of the canine hippocampus, as in humans, but not in the other subregions or the entorhinal cortex of aged dogs. Stereological studies in humans report a loss of neurons in the hilus and subiculum of the hippocampus (70,89,92) with normal aging. Some animal models show hilar neuron loss while others do not. Our data from the dog indicates that neuron loss occurs with age in the hilus of the hippocampus, similar to humans. Our results are limited to the left hemisphere of the brain. The age-related loss of neurons in the hilus is likely a result of increased cell death with age (42) possibly due to beta-amyloid deposition (34,84), oxidative damage (35), DNA damage (4) or cerebrovascular damage (79). A reduction in hippocampal neurogenesis with age is not likely to account for the loss of neurons observed in the present study since the hilus is not thought to be a neurogenic region in the dog (71). Aged rats also lose hilar neurons but this loss may not be apparent until after 22 months of age (7,11,67). In contrast, non-human primate models of aging have not found an equivalent loss of neurons with normal aging in the hippocampus (41,90).

The hilus contains a heterogeneous population of neurons that connect to the granule cell layer and other hippocampal fields (52). Hilar neurons are vulnerable to many different insults and events and loss is observed in several animal models after exposure to excitotoxins (6), ischaemia (98), stress (19), hypertension (57), and diabetes mellitus (9). The loss of hilar neurons can be ameliorated with treatments including growth hormone (7), fluoxetine (9), substance P receptor antagonist (19), and estradiol (6). Hilar neuron damage is also characteristic of temporal lobe epilepsy (20). Patients with temporal lobe epilepsy exhibit deficits in declarative memory and visuospatial task performance (1,27,28,38). Memory and learning deficits are frequently observed in animals with hilar neuron damage (98) but since the damage usually extends to regions outside of the hilus it is difficult to isolate the precise role of the hilus in learning and memory functions.

Neuron loss in the entorhinal cortex in normal human aging is not detectable when each complete layer of the entorhinal cortex is examined (93), but has been reported in studies where the counts are limited to the islands of layer II (37,69). Neuron loss in the entorhinal cortex is more commonly observed in cognitively impaired older people (43) and Alzheimer's disease patients (58). Von Gunten et al. (88) reported that dementia in extreme aging (over 90 years of age) may involve damage to the hippocampal subdivisions with a relative sparing of the entorhinal cortex and CA1 fields. Studies in non-human primates (46) and rats (45) have not found a loss of entorhinal neurons with age in any layer but none have examined the islands specifically. The present study found no evidence for neuron loss in the entorhinal cortex or specifically layer II in the dog, which is consistent with normal aging in humans and with other animal models.

4.2 Reducing age-associated neuron loss in the hilus

We compared the effects of two interventions on neuron survival that were each hypothesized to lead to a preservation of neuron number with age in dogs. The first treatment involved behavioral enrichment, which in rodent studies leads to increased neurogenesis (40) and reduces neuron loss in experimental Parkinsonism (22). The second intervention was to provide animals with a food rich in a broad spectrum of antioxidants that may serve to maintain healthy function and reduce neuron death associated with aging. Based on studies in rats demonstrating that vitamin E regulates neurogenesis and supplementation reduces apoptotic cell death (12, 15,18,23), we hypothesized that the combination of behavioral enrichment and an antioxidant fortified food may lead to higher neuron numbers than either treatment alone. The results of the current study suggest that the behavioral enrichment intervention, but not the antioxidant fortified food, selectively increased hilar neuron numbers in aged animals relative to age-matched controls. The treatment effect was relatively small however, and may be due to the age at which the intervention was initiated. It is possible that a larger treatment effect would occur had the animals started the intervention at a younger age or if the treatment duration was extended. Olson et al. (55) recently showed that environmental enrichment increases the likelihood of survival of new cells in the dentate gyrus. This suggests that the effect of behavioral enrichment in the canine hilus may be neuroprotective and acts by reducing cell death in the dog brain.

The antioxidant fortified food used in the current study did not modify neuron numbers in the canine hilus. Our previous work indicates that the antioxidant fortified food does, however, reduce beta-amyloid accumulation in the parietal cortex (60) possibly by increasing the activity of the alpha-secretase enzyme (59), which cleaves the amyloid precursor protein and prevents the production of beta-amyloid. The behaviorally enriched animals showed no evidence of reduced beta-amyloid. These data suggest that the behavioral enrichment and antioxidant fortified food may have unique or possibly independent neurobiological mechanisms for enhancing cognitive function (48,49,50,51,73). Here we report a beneficial effect selective for

behavioral enrichment on neuron number whereas our previous work suggested a reduction in beta-amyloid selectively occurs in the antioxidant treated animals (59,60).

4.3 Neuron loss and cognition

The hippocampus is involved in learning and memory processes that decline with age (21) and neuron loss is a potential morphological correlate of cognitive dysfunction. We have previously demonstrated age-related declines in the dog affecting several cognitive domains including memory, complex visuospatial learning and executive functions (36,47,81,82,83,85). We did not observe a significant relationship between errors on the spatial memory or discrimination learning tasks assessed during the treatment phase of the study and neuron number in the hilus. This is consistent with studies in aged rats where entorhinal cortex and hippocampal neuron numbers do not correlate with spatial learning and memory (11,45,62,63). This suggests that the reduction in hilar neuron loss does not account for the improved learning and memory in the treated aged dogs.

Although neuron loss may contribute to cognitive decline, other factors must be important since cognitive decline is detectable prior to overt neuron loss and few tasks correlate with measures of neuron number. Other events leading to neuronal dysfunction such as oxidative damage (35), DNA damage (4), the presence of extensive beta-amyloid pathology (34,84), or cerebrovascular changes (79) may be contributors to cognitive decline in aging dogs. Synapse loss may also contribute to cognitive decline (16) but this has not yet been examined in the dog.

5. Conclusions

The aged canine hippocampus is vulnerable to neuron loss in the hilus. Age-related losses of hilar neurons in the canine may be reduced by behavioral enrichment but do not appear to be significantly affected by antioxidant treatment. These results extend previous studies in rodents to a higher mammal with consistent results. Although both treatment conditions improved cognition in aged canines, the changes in neuronal function seem to be mediated through independent molecular pathways.

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Figure 1. Relative plots of cell position within the tissue sections for each group of dogs. The plots appear similar between the groups.

Figure 2.
A montage showing the NeuN immunohistochemical stained canine hippocampus with subregions and the entorhinal cortex with layer II delineated for neuron counts.

Figure 3. Hilar neuron number in aged dogs is significantly lower compared to young dogs ($p = .012$). Hilar neuron number in aged dogs provided with behavioral enrichment was significantly higher compared to aged control dogs ($p = .041$).

Table 1

Experimental parameters used with the optical fractionator: neuron count = ΣQ^- ; area of the disector frame = a(frame); area sampling fraction = asf; section thickness = t; height of the optical disector = h; thickness sampling fraction = tsf; section sampling fraction = ssf.

	mean $\Sigma Q^- \pm$ SD	A(frame) (μm^2)	x,y step ($\mu\text{m} \times$ μm)	asf	mean t $\pm$ SD (μm)	h (μm)	tsf	mean number of sections $\pm$ SD	ssf
Granule	955 $\pm$ 217	716.0	300 $\times$ 300	.0080	19.43 $\pm$ 0.95	t	1	11 $\pm$ 2	1/24
Hilus	371 $\pm$ 89	1432.1	150 $\times$ 150	.0636	19.43 $\pm$ 0.95	t	1	11 $\pm$ 2	1/24
pCA3	597 $\pm$ 84	1432.1	150 $\times$ 150	.0636	19.43 $\pm$ 0.95	t	1	10 $\pm$ 2	1/24
dCA3	299 $\pm$ 31	1432.1	250 $\times$ 250	.0229	19.43 $\pm$ 0.95	t	1	10 $\pm$ 2	1/24
CA2	318 $\pm$ 60	1432.1	150 $\times$ 150	.0636	19.43 $\pm$ 0.95	t	1	11 $\pm$ 2	1/24
CA1	259 $\pm$ 37	1432.1	600 $\times$ 600	.0040	19.43 $\pm$ 0.95	t	1	11 $\pm$ 2	1/24
Subiculum	273 $\pm$ 33	1432.1	500 $\times$ 500	.0057	19.43 $\pm$ 0.95	t	1	10 $\pm$ 2	1/24
Entorhinal	128 $\pm$ 32	1432.1	800 $\times$ 800	.0022	19.43 $\pm$ 0.95	t	1	10 $\pm$ 2	1/24
Layer II	316 $\pm$ 31	1432.1	200 $\times$ 200	.0358	19.43 $\pm$ 0.95	t	1	10 $\pm$ 2	1/24
Treatment Study									
Hippocampus	172 $\pm$ 36	1432.1	1700 $\times$ 1700	.0005	19.13 $\pm$ 1.03	t	1	13 $\pm$ 2	1/24
Entorhinal	120 $\pm$ 32	1432.1	800 $\times$ 800	.0022	19.13 $\pm$ 1.03	t	1	10 $\pm$ 2	1/24
Hilus	311 $\pm$ 57	1432.1	150 $\times$ 150	.0636	19.13 $\pm$ 1.03	t	1	11 $\pm$ 2	1/24

All evaluations were performed with a 60x oil objective lens (N.A. 1.4)

Table 2

Estimated individual unilateral neuron number ($N \times 10^3$) with CE in each subregion of the hippocampal formation and entorhinal cortex of young beagle dogs: mean group numbers (mean N), standard deviation (SD), coefficient of error (CE and mean CE).

Age (yrs)	Granule		hilus		pCA3		dCA3		CA2		CA1		Subiculum		Sum		Entorhinal		Layer II	
	N	CE	N	CE	N	CE	N	CE	N	CE	N	CE	N	CE	ΣN	ΣN	N	CE	N	CE
3.4	2554	.0473	184	.0503	236	.0501	366	.0527	140	.0665	1255	.0715	1134	.0680	5841	1384	.0916	.0916	229	.0611
4.1	3826	.0392	160	.0588	188	.0496	287	.0649	102	.0668	1918	.0590	1292	.0688	7639	1384	.0916	.0916	191	.0639
4.2	2803	.0380	191	.0467	260	.0423	289	.0687	130	.0569	1490	.0643	1028	.0779	6161	2132	.0798	.0798	231	.0589
4.4	3561	.0389	145	.0589	233	.0501	308	.0621	123	.0617	1786	.0613	1294	.0660	7441	1437	.0896	.0896	193	.0646
4.5	4220	.0355	140	.0575	247	.0446	290	.0645	113	.0667	1356	.0701	953	.0738	7234	1106	.1010	.1010	196	.0642
Mean	3393	.0399	164*	.0546	233	.0474	308	.0628	122	.0638	1561	.0654	1140	.0710	6863	1488	.0909	.0909	208	.0602
SD	699		23		27		33		15		282		153		808		383		20	
CV ²	.0424		.0201		.0135		.0117		.0145		.0327		.0181				.0660		.0094	
CE ²	.0016		.0030		.0023		.0039		.0041		.0043		.0050				.0083		.0036	
BCV ²	.0408		.0172		.0112		.0077		.0104		.0284		.0131				.0578		.0058	
(% of CV ²)																				
BCV ²	96		85		83		66		72		87		72				88		62	

BCV² = CV² - CE² (CE = coefficient of error; CV = coefficient of variation; BCV = biological coefficient of variation)

* significantly higher than aged dogs (p < 0.05)

Table 3

Estimated individual unilateral neuron number ($N \times 10^3$) with CE in each subregion of the hippocampal formation and entorhinal cortex of aged beagle dogs: mean group numbers (mean N), standard deviation (SD), coefficient of error (CE and mean CE).

Age (yrs)	Granule		hilus		pCA3		dCA3		CA2		CA1		Subiculum		Entorhinal		Layer II	
	N	CE	N	CE	N	CE	N	CE	N	CE	N	CE	N	CE	N	CE	N	CE
13.0	2516	.0412	96	.0688	206	.0480	281	.0646	112	.0633	1351	.0691	1047	.0683	1502	.0869	237	.0592
13.6	4248	.0315	107	.0616	246	.0423	348	.0575	167	.0497	1520	.0663	1303	.0617	1856	.0776	206	.0611
14.2	3442	.0352	158	.0525	256	.0438	358	.0569	109	.0614	1376	.0684	1123	.0675	1062	.1030	244	.0561
14.5	2929	.0364	108	.0637	218	.0464	291	.0619	119	.0587	1725	.0613	1127	.0660	1523	.0853	196	.0607
15.0	2371	.0444	110	.0671	161	.0547	309	.0656	83	.0729	1750	.0659	1341	.0646	697	.1270	197	.0637
Mean	3101	.0380	116*	.0630	217	.0473	318	.0614	118	.0617	1544	.0663	1188	.0657	1328	.0976	216	.0626
SD	764		24		37		34		30		188		127		451		22	
CV ²	.0607		.0428		.0296		.0114		.0664		.0148		.0114		.1156		.0111	
CE ²	.0014		.0039		.0022		.0038		.0038		.0044		.0043		.0095		.0039	
BCV ²	.0593		.0389		.0274		.0077		.0626		.0104		.0071		.1060		.0072	
(% of CV ²)																		
BCV ²	98		91		93		67		94		70		62		92		65	

BCV² = CV² - CE² (CE = coefficient of error; CV = coefficient of variation; BCV = biological coefficient of variation)

* significantly lower than young dogs (p < 0.05)

Table 4

Correlations between neuron counts in the hilus of the hippocampus and error scores on cognitive tasks.

Task	Timepoint	r	p
Spatial Memory	1 year	-0.266	0.243
Size Discrimination	1.5 years	-0.464	0.030
Size Reversal	1.5 years	-0.332	0.132
Spatial Memory	2 years	-0.184	0.438
Black/White Discrimination	2.5 years	0.016	0.946
Black/White Reversal	2.5 years	-0.348	0.122

p < 0.0083