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Aged dogs demonstrate both increased sensitivity to scopolamine impairment and decreased muscarinic receptor density

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ABSTRACT

Memory deficits associated with aging and Alzheimer's disease have been linked to cholinergic dysfunction. The present study investigated this hypothesis by comparing the effects of the muscarinic cholinergic receptor antagonist scopolamine on recent memory performance and by examining muscarinic receptor density in aged and young dogs. Scopolamine (15 μg/kg; SC) was administered prior to testing young (M = 2.8 years) and aged (M = 13.0 years) dogs on a delayed-non-matching-to-position task (DNMP). Scopolamine significantly impaired performance of aged, but not young dogs. Muscarinic receptor density was assessed autoradiographically using the non-selective radioligand [^{3}H]quinuclidinylbenzilate. Aged dogs (M = 14.1 years) showed significantly decreased density of muscarinic receptors in all brain regions examined except the cerebellum compared to young dogs (M = 3.7 years). The results are consistent with those seen in aged humans and Alzheimer's patients and support the hypothesis of age-dependent cholinergic dysfunction in the dog, although this was not directly determined in the current study. These findings demonstrate that markers of cholinergic hypofunction, in addition to the natural cognitive decline and amyloid pathology previously noted, are seen in canine aging. Collectively, this supports the use of the aged dog as a model for examining early pathological events in the development of Alzheimer's disease.

1. Introduction

Alzheimer's disease is a neurodegenerative condition of humans initially characterized by decline in episodic memory that progressively develops into a global dementia. Although the cause of disease remains unknown, deposition of amyloid-β into senile plaques and the formation of neurofibrillary tangles within the brain are both hallmark features that are considered potential therapeutic targets (Jakob-Roetne and Jacobsen, 2009). Particular emphasis has been placed on the amyloid cascade hypothesis (Hardy and Higgins, 1992) in current drug development, but, to date, the greatest clinical success has been shown by therapeutic strategies targeting cholinergic dysfunction, which is thought to play an important role in memory decline in both aging and Alzheimer's disease (Bartus et al., 1982). The cholinergic hypothesis was originally based on a convergence of evidence from pharmacological, biochemical and clinical studies suggesting that a severe reduction in cholinergic function contributed to the cognitive symptomology associated with advanced age and Alzheimer's disease (Bartus, 2000). Patients with Alzheimer's disease show reduced post-mortem activity levels of choline acetyltransferase (CAT; Davies and Maloney, 1976; Perry et al., 1977), an inverse correlation between CAT activity and pre-morbid intellectual impairment (Perry et al., 1978), and selective degeneration of the cholinergic nucleus basalis of Meynert (Whitehouse et al., 1981), which projects extensively to the cortex. The cholinergic hypothesis is also supported by pharmacological evidence that scopolamine, a non-selective muscarinic cholinergic antagonist, impairs the cognitive ability of young humans to levels seen in drug-free non-demented elderly subjects (Drachman and Leavitt, 1974) and impairs cognitive performance in the elderly to a greater extent than in the young (Flicker et al., 1992; Ray et al., 1992).

Although the cholinergic hypothesis was instrumental in the rational development of the first approved drugs for Alzheimer's disease, the cholinesterase inhibitors, there is substantial controversy over the role of cholinergic dysfunction in cognitive impairment (e.g. Bartus, 2000; Decker and McGaugh, 1991; Fibiger et al., 1991; Nobili and Sannita, 1997). Muscarinic receptor density, measured in post mortem and in vivo imaging studies (Norbury et al., 2005; Reinikainen et al., 1987; Rinne et al., 1985; Zubieta et al., 2001), and sensitivity to scopolamine impairment (Flicker et al., 1992; Huff et al.,

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1988; Sunderland et al., 1987) is intermediate in the aged compared to young controls and AD patients, supporting a progressive decline in cholinergic function across aging to Alzheimer's disease. Moreover, studies in aged humans and animals suggest that cholinergic hypofunction is probably a correlate of aging, but markers of robust cholinergic cell loss are less evident in aged populations compared to Alzheimer's patients suggesting the more robust loss of cholinergic cells occurs late in disease progression (Contestabile et al., 2008; Decker, 1987; McGeer et al., 1984). While non-selective immunotoxic lesions of the basal cholinergic forebrain result in memory impairments in rodents and non-human primates, more selective immunotoxins (i.e. 192 IgG-saporin) that spare non-cholinergic neurons produce more specific cognitive impairments, which may largely be limited to disruption of attentional processes (Gallagher and Colombo, 1995; McGaughy et al., 2000; Muir, 1997), but can occasionally affect memory functions (e.g. Lehmann et al., 2003). Therefore, cholinergic deficits alone cannot account for the totality of cognitive impairments seen in Alzheimer's disease. In this context, it is not surprising that the current cholinesterase inhibitors have limited efficacy and show substantial individual variability when used for treatment of Alzheimer's disease (Birks, 2006; Doody, 2003; Pepeu and Giovannini, 2009). Complicating the issue, recent studies show that the cholinergic system is highly susceptible to amyloid toxicity and that amyloid processing can be altered by cholinergic stimulation (Fisher, 2008; Pakaski and Kalman, 2008), which suggests a potential bidirectional interaction between both cholinergic and amyloid pathological events (Hellstrom-Lindahl, 2000).

Dogs show several changes with aging that makes them well suited for testing hypotheses and therapeutics related to the development and treatment of Alzheimer's disease (Cotman and Head, 2008; Woodruff-Pak, 2008). Dogs exhibit a specific pattern of age-dependent cognitive decline (Adams et al., 2000a) including impairment on tests of complex discrimination learning (Milgram et al., 2002a, 2002b), reversal learning (Milgram et al., 1994; Tapp et al., 2003a), object recognition memory (Callahan et al., 2000; Milgram et al., 1994), and short-term visuospatial working memory (Adams et al., 2000b). By contrast, simple procedural memory and discrimination learning is generally spared with increasing age (Head et al., 1995). Like humans, individual aged dogs show a spectrum of intact to impaired cognitive function allowing grouping into distinctive categories of normal aged, mildly impaired and severely impaired groups similar to the heterogeneity and classifications seen in humans (Adams et al., 2000a; Cotman and Head, 2008). Impairment in the dog also is associated with Alzheimer's-like neuropathological changes, particularly the deposition of amyloid-β, which is identical in protein sequence and undergoes similar post-translational modifications as that seen in humans (Cummings et al., 1996; Cotman and Head, 2008; Head et al., 1998). The chronological pattern of brain deposition of amyloid-β into diffuse plaques is also similar to that seen in humans, probabilistically increases with age (Head et al., 2000), and has been regionally linked to deficits on specific cognitive tasks (Cotman and Head, 2008; Tapp et al., 2004). The relative levels and number of amyloid-β isoforms in CSF of aged dogs is similar to that seen in Alzheimer's patients (Portelius et al., 2010). Tau hyperphosphorylation also occurs in dogs (Pugliese et al., 2006; Wegiel et al., 1998), but neurofibrillary tangles are not evident (but see Papaioannou et al., 2001 for an opposite finding), possibly due to the life-span of the dog or differences in tau protein sequence (Cotman and Head, 2008). Given the absence of full-blown Alzheimer's pathology and the heterogeneity of cognitive decline seen in aged dogs, Cotman and Head (2008) suggest that the aged dog models the spectrum from normal aging through mild cognitive impairment (MCI) and early/mild Alzheimer's disease, which should be particularly useful for testing hypotheses and therapeutics targeted at reducing or preventing conversion of early cognitive impairments to Alzheimer's disease.

An important limitation of the aged canine model is the absence of studies examining neurochemical changes that occur during aging and their role in cognitive decline (Woodruff-Pak, 2008). We previously obtained evidence of cholinergic mediation in canine cognitive function in aged dogs (Araujo et al., 2004). Administration of scopolamine to aged dogs causes impaired performance on a visuospatial working memory task, the delayed-non-matching-to-position task (DNMP), at a dose of 15 μg/kg; doses of 5 or 10 μg/kg had no effect. By contrast, a 15-μg/kg dose had no effect on: a landmark discrimination test of allocentric discrimination ability; on previously learned discrimination performance; and on general behavioral measures assessed in the open field. In humans and non-human primates, delayed tasks are particularly sensitive to scopolamine disruption, increasing age and cognitive status, presumably because of progressive cholinergic decline (Bartus, 2000). Performance on the DNMP is impaired relatively early in canine aging (Studzinski et al., 2006). Collectively this suggests that dogs may demonstrate cholinergic decline with aging; however, studies examining cholinergic markers in aged dogs compared to young are lacking. Given the natural development of amyloid pathology in aged dogs, evidence of cholinergic dysfunction would suggest the aged dog is well suited for examining the interaction between these two pathological events in age-related cognitive decline.

The present experiment used two strategies to examine the effect of age on markers of cholinergic function in canine aging. The first employed a functional strategy similar to that described previously in which we compared the effects of the threshold 15 μg/kg dose of scopolamine on DNMP performance in both young and aged dogs. We hypothesized and found that aged dogs were more sensitive to the scopolamine-induced deficits similar to the data obtained in humans (Flicker et al., 1992; Ray et al., 1992). To explore whether this functional difference might be attributed to physiological changes in the cholinergic system, we then examined the effects of age on muscarinic receptor density using autoradiography in archived canine brain tissue. We hypothesized that aged dogs would show reduced levels of muscarinic receptor density similar to studies conducted in aged and demented humans (Norbury et al., 2005; Reinikainen et al., 1987; Rinne et al., 1985; Zubieta et al., 2001).

2. Materials and methods

2.1. Subjects

The subjects consisted of cognitively experienced Beagle dogs that were maintained in the canine colony at the University of Toronto. Four male and 3 female aged dogs between the age of 10.2 and 14.2 ($M = 13.0 \pm 1.41$) years and 5 male and 2 female young dogs between the age of 1.9 and 3.8 ($M = 2.71 \pm 0.81$) years were tested on a 3-component DNMP (Chan et al., 2002) task. All of the subjects of this colony underwent a cognitive training program and were included in a larger longitudinal study examining age-related cognitive function. The subjects were housed individually, or in pairs, in pens measuring approximately 1.07×1.22 m. Males and females were housed separately as were aged and young dogs. The dogs were fed once daily in the afternoon with Canine Laboratory Chow #5006 (Purina Agribrands, Woodstock, ON), had access to water *ad libitum* and were exercised daily while their pens were cleaned. Clinical veterinary assessment of the animals in the study precluded the use of dogs with any health, neurological or sensory deficits that could interfere with cognitive testing. Similarly, no subject showed abnormalities in clinical blood measures, which were obtained on a yearly basis. All procedures were conducted in accordance with the "Principles of laboratory animal care" (NIH publication No. 85–23, revised 1996), the guidelines of the Canadian Council on Animal Care and were approved by the University of Toronto Local Animal Care Committee. The dogs were returned to the colony following this study for inclusion in other studies.

2.2. Cognitive test apparatus

Subjects were tested in a canine adaptation of the Wisconsin General Test Apparatus as described previously (Milgram et al., 1994). The apparatus was a wooden box measuring $0.61 \times 1.15 \times 1.08$ m. Adjustable vertical steel bars enclosed the front of the box and provided access to the test stimuli and rewards. For all tests, the reward consisted of approximately one-cubic centimeter of a moist canine diet. A sliding Plexiglas tray, with food wells in the right, center and left position, was used to present the stimuli to the dogs. The stimuli were placed over the food wells and a food reward was placed in the well under the rewarded stimulus. A wooden partition with a hinged door, that permitted presentation of the tray when opened, and a one-way mirror separated the tester and subject. An incandescent light located at the front of the box was the only light source during all testing procedures and enabled the experimenter to observe the subjects undisturbed during testing.

2.3. Effect of scopolamine on memory performance in the variable DNMP

Dogs were selected for this experiment based on both ability to perform the task and age. Dogs less than 5 years of age were selected for the young group because this age range is unlikely to demonstrate impairment on the task (Studzinski et al., 2006). Dogs with high levels of task experience and performance accuracy greater than 10 years of age were selected for the aged group consistent with the age grouping of Cotman and Head (2008). Each trial on the DNMP task consisted of 2 phases. During the first phase, a red block was presented to the animal over one of the three food wells. Once the animals displaced the block and obtained the hidden food reward, the tray was withdrawn and a delay of 5, 55 or 105 s was initiated. Following the delay, two identical red blocks were presented to the subject; one was located over the same food well as in the first phase and the second was over one of the 2 other food wells. Displacement of the block in the new, or non-match, position revealed the food reward beneath. Each delay occurred exactly six times during each session, and the position of the correct response was varied such that each position was correct on exactly one-third of the total number of trials. To prevent the animals from using olfactory cues to solve the task, a quantity of the rewarded food approximately equal to that associated with the non-match was smeared on the bottom of the incorrect stimulus. A one-minute inter-trial interval (ITI) separated the 18 daily trials.

Prior to scopolamine testing, all subjects received 9 daily test sessions to ensure stabile performance. Scopolamine testing then occurred in four-day blocks consisting of a control (no treatment), vehicle-treatment, scopolamine-treatment (15 μg/kg, SC), and a drug-washout day. This cycle was repeated for a total of 15 days (no wash-out day was provided on the last block) and testers were blind to the drug condition. The design was chosen to limit potential carry-over effects of scopolamine on cognitive performance the following day, which has not been effectively characterized in the dog.

Scopolamine hydrobromide was obtained from Sigma-Aldrich Inc. The drug was prepared for administration in a 100-μg/ml base solution by dissolution in 0.9% normal saline and was administered subcutaneously one hour prior to testing at a dose of 15 μg/kg. Vehicle injections consisted of an equivalent volume of 0.9% normal saline.

2.4. Brain tissue

Frozen (−80 °C) brain tissue from 3 male and 3 female young subjects between 2.2 and 7.1 ($M = 3.67 \pm 1.92$) years and 3 male and 3 female aged subjects between 10 and 15.7 ($M = 14.08 \pm 2.20$) years was obtained from archived canine brain tissue. All dogs were originally from the University of Toronto colony, had some level of previous cognitive test experience, but were not cognitively tested prior to euthanasia, which precluded our ability to correlate cognitive status with muscarinic receptor density immediately prior to death. The tissue from these dogs was selected due to the exploratory nature of the experiment, which did not warrant use of tissue from dogs better characterized cognitively. One dog in the young group was 7.1 years of age, representing a middle aged dog (Cotman and Head, 2008), but the exclusion of this dog resulted in a population of dogs between 2.2 to 4.5 ($M = 3.0 \pm 1.1$) years of age. Thus age groups between experiments were similar, but these dogs represented slightly older dogs than those used in the scopolamine experiment; other measures of Alzheimer's pathology were not conducted on this tissue. The tissue was harvested similarly for all subjects as described previously and within one-half hour of death (Head et al., 2000). Thirty-six consecutive 20 μm cryostat sections (~−21 °C) were taken from each of the following areas: frontal cortex; parietal cortex; entorhinal cortex; occipital cortex; and cerebellum; as per previous experiments examining canine brain pathology (Head et al. 2000). Sections were mounted on FisherBrand Superfrost/Plus slides and maintained at −80 °C until the autoradiographic analysis. In the present experiment, every sixth section was used for analysis (i.e., every 100 μm was examined per brain area).

2.5. Quantitative muscarinic receptor binding

The non-selective muscarinic ligand [^{3}H]quinuclidinylbenzilate ([^{3}H]QNB) was used to quantify muscarinic receptors. The tissue sections were brought to room temperature and preincubated in 10 mM PBS buffer pH 7.4 at 4 °C for 30 min. The slides then were incubated in a 1 nM [^{3}H]QNB (Amersham, Buckinghamshire, UK, 42 Ci/mmol) buffer solution for 60 min at room temperature. Non-specific binding was defined by the addition of 1 μM atropine to adjacent slices. The slides were rinsed twice for 5 min in ice-cold 10 mM PBS buffer and rapidly dipped in cold deionized H_2O following the incubation. Slides then were dried under a stream of cold air and exposed in the presence of calibrated standards to ^{3}H-sensitive film (Hyperfilm, Amersham, Buckinghamshire, UK) for 4 weeks. Densitometric analyses were conducted on the developed film using an AIS/MCID system (Imaging Research, St. Catharine's, ON. Canada) and the final binding value for each structure was the average of multiple readings on all six of the sections.

2.6. Analysis

The DNMP data were analyzed using a repeated-measures analyses of variance (ANOVA) with age group serving as a between-subject variable, and treatment (control, saline and scopolamine) and delay (5, 55 and 105) serving as within-subject variables. Mean percentage of correct responses compared to total trials (i.e. accuracy) at each delay served as the dependent variable. Post-hoc Fisher's test was used to evaluate main effects and interactions when appropriate. To analyze the differences in muscarinic receptor levels, we used an identical approach to a similar study conducted in humans in which a two-tailed independent *t* test was used to determine group differences for each brain region tested (Rodriguez-Puertas et al., 1997). Statistical analysis was conducted with the Statistica 6.0 (Statsoft Inc.) software package with the significance level set to $p<0.05$.

3. Results

3.1. Effect of scopolamine on visuospatial working memory in young and old dogs

Analysis of the DNMP results revealed a significant main effect of treatment [$F(2,24) = 14.05$, $p<0.001$], due to significantly decreased performance under scopolamine compared to both control and saline conditions [$p<0.05$ in both cases], and a significant age by treatment

interaction [$F(2,24) = 3.61$, $p<0.05$]. Post-hoc Fisher's revealed that performance of aged dogs after scopolamine was significantly poorer than performance under control [$p<0.05$] and saline [$p<0.01$] conditions. Performance under control did not differ from performance under saline. By contrast, no treatment effects were found in the young dogs (Fig. 1b). However, scopolamine performance of aged dogs differed significantly from control performance [$p<0.05$] and marginally from saline performance [$p<0.08$] of young dogs, but differences between groups on scopolamine performance were not seen [$p>0.35$]. There was also a main effect of delay [$F(2,24) = 31.44$, $p<0.001$], due to decreasing performance accuracy of both old and young groups with increasing delay, which is consistent with previous findings (Araujo et al., 2004). Fisher's post-hoc analysis indicated that the medium and long delays both differed significantly from the shortest delay [$p<0.05$ in both cases], but not from each other [$p<0.11$]. An interaction between delay and treatment was not found [$p>0.4$] indicating that scopolamine did not differentially suppress performance at any delay. No difference in control or saline performance was found between the groups.

3.2. Quantitative muscarinic receptor binding

As has previously been reported (Hamann et al. 2006), muscarinic binding was found throughout the cortex and was abolished by atropine. Four intensity layers were present in the frontal cortex, 2 in the cerebellum, temporal and parietal cortices, and 3 in the occipital cortex. For analysis, mean density of all samples in each region was used. Independent *t* tests indicated significantly reduced muscarinic receptor density in aged dogs compared to young dogs in frontal, parietal and entorhinal cortices (Table 1). The largest overall differences in muscarinic receptor density was found in the occipital lobe between young and aged dogs (15.2%), but this difference was marginally significant [$p = 0.07$] likely due to higher levels of variability in this region compared to the others. The only region that did not show an age-dependent decrease in muscarinic receptor binding was the cerebellum, which is considered a control region for amyloid pathology (Head et al., 2000). No gender differences were found.

Table 1
[^{3}H]QNB binding to muscarinic receptors in aged and young dog brains[a].

Brain region	Aged (N = 6)		Young (N = 6)		p[b]
	Mean	S.D.	Mean	S.D.	
Frontal	5.36	0.37	6.04	0.41	0.013
Parietal	4.59	0.34	5.29	0.18	0.0012
Entorhinal	5.28	0.41	5.76	0.18	0.026
Occipital	4.69	0.92	5.53	0.43	0.07
Cerebellum	1.68	0.23	1.66	0.12	0.84

[a]Values are expressed in fmol/mg tissue.
[b]Two-tailed independent *t* tests.

4. Discussion

The current studies were aimed at investigating the effect of canine aging on two cholinergic markers previously shown to be altered in studies of human aging and Alzheimer's disease. The first experiment used a pharmacological approach to measure functional differences in young and aged dogs, and similar to studies in humans (Flicker et al., 1992; Ray et al., 1992) and rodents (Biggan et al., 1996; Stemmelin et al., 1999), we found that aged dogs were more sensitive to scopolamine-induced DNMP deficits than young dogs. To explore whether this age-dependent hypersensitivity to scopolamine might be related to an age-dependent physiological change in the cholinergic system, we then examined muscarinic receptor density, which has been shown to decline with age and in Alzheimer's disease in humans (Norbury et al., 2005; Reinikainen et al., 1987; Rinne et al., 1985; Zubieta et al., 2001), in archived brain tissue of aged and young dogs. Compared to tissue from young dogs, significantly decreased muscarinic receptor density was found in all areas of the aged brain with the exception of the occipital cortex and cerebellum. The use of different populations of dogs in the two experiments, in addition to other limitations discussed below, precludes us from concluding that the hypersensitivity to scopolamine seen in aged dogs is due to age-dependent alterations in the cholinergic system. However, the results of the current studies strongly support this possibility, particularly when considered in conjunction with previous findings in other species. To the best of our knowledge, this is the only study that provides evidence that the cholinergic system may undergo significant changes with aging in dogs, which warrants a more thorough characterization of age-dependent cholinergic changes in canine aging and cognitive decline.

The first experiment showed that DNMP performance in aged, but not young dogs, was impaired by scopolamine using the previously established minimal effective dose for producing selective impairment on this task in aged dogs (Araujo et al., 2004). The absence of differences in control performance between the young and aged group is not consistent with previous findings (Chan et al., 2002; Studzinski et al., 2006), which suggests the selection criterion used in the current study resulted in a subpopulation of highly functioning aged dogs. However, five of the seven aged dogs used in this study and four of the seven young dogs were included in a previous study demonstrating age-related impairments on a working memory task with greater cognitive demands than the DNMP task (Tapp et al., 2003b). Thus, the absence of age differences in control DNMP performance in the current study is more likely attributed to differential experience of the two groups (Milgram, 2003; Milgram et al., 2006) and the aged group in the current study likely represents

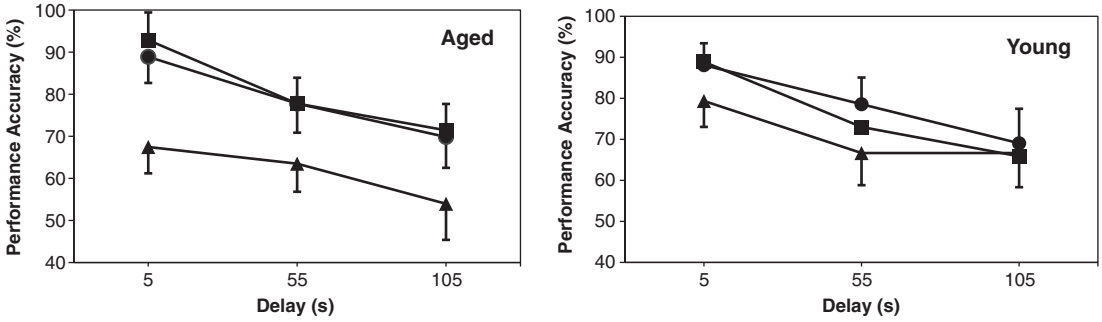

Fig. 1. Mean DNMP performance accuracy of aged and young dogs following control (circle), saline vehicle (square) and 15 μg/kg scopolamine (diamond). Animals were tested at delays of 5, 55 and 105 s. Aged dogs were significantly impaired by scopolamine compared to their control and vehicle performance. Young dogs were not impaired by scopolamine compared to their control or vehicle performance. No delay-dependent scopolamine effects were found. Error bars represent the standard error of the mean.

a subset of aged dogs with mild or selective cognitive impairment rather than successful agers. Therefore, scopolamine hypersensitivity may be apparent in populations of aged dogs that may correspond to the human mildly impaired or MCI population (Cotman and Head, 2008), suggesting that decreased cholinergic function may occur relatively early in age-dependent canine cognitive decline.

While scopolamine hypersensitivity in aged and demented humans is suggested to represent a pharmacological measure of age-related cholinergic dysfunction (Sunderland et al., 1987), the absence of pharmacokinetic measures and functional comparisons with quaternary amine derivatives of scopolamine confounds this interpretation. Quaternary amines, which don't readily cross the blood-brain barrier, are used to differentiate peripheral from central anticholinergic effects on task performance; however, this approach has revealed inconsistent results in other species (Moore et al., 1992). The effectiveness of this approach would be limited in the current study because aged dogs demonstrate blood-brain barrier deterioration (Su et al., 1998). Consequently, we elected to use a previously established threshold dose of scopolamine that selectively impaired DNMP performance in aged dogs while sparing performance on other tasks requiring similar behavioral resources to limit the influence of peripheral anticholinergic effects (Araujo et al., 2004). Similarly, the absence of delay-dependent effects seen in the current study could reflect general behavioral impairment related to peripheral anticholinergic effects, but the absence of delay dependency is consistent with our previous study using this threshold scopolamine dose (Araujo et al., 2004) and, therefore, more likely reflects scopolamine-induced attentional impairments, which has been suggested by others (Broks et al., 1988; Cheal, 1981; Chen et al., 2004; Davidson et al., 1999; Dunne and Hartley, 1986; Furey et al., 2008; Jones and Higgins, 1995; McGaughy et al., 1994). The absence of pharmacokinetic measures is potentially more problematic because decreased liver function and blood-brain barrier integrity is common in the aged and can ultimately result in higher levels of scopolamine exposure. This confound is unlikely to fully account for the age differences in scopolamine sensitivity seen in the current study for several reasons. First, the dogs used in the current study did not show clinical signs consistent with liver changes. Second, the subcutaneous route of injection is expected to circumvent the large first-pass effects expected with scopolamine (Renner et al., 2005), which further limits the potential impact of non-clinical age-related differences in liver function. Third, scopolamine is highly lipophilic and readily crosses the blood-brain barrier, which reduces the likelihood that scopolamine brain penetration was higher in aged dogs. Fourth, sensitivity to scopolamine in elderly humans and Alzheimer's patients is not related to differences in plasma levels of scopolamine, supporting the hypothesis that differences in sensitivity are due to reduced cholinergic function rather than pharmacokinetic differences (Sunderland et al., 1987; 1988). Last, studies using various methods for assessing cholinergic integrity repeatedly demonstrate age-related deficiencies across species (Bartus, 2000; Decker, 1987; Markowska et al., 1995), and it is unlikely that the dog differs in this respect. While future studies examining the metabolism of scopolamine in canine aging are warranted, the age differences seen in the current study are likely attributed, at least in part, to decreased cholinergic function with age.

In an attempt to partially explain the hypersensitivity of scopolamine in aged dogs and to examine the possibility that canine aging is associated with physiological alterations of the central cholinergic system, the non-selective radioligand, [^{3}H]QNB was used to examine muscarinic receptor density in both young and aged dogs. Due to the exploratory nature of this study, we cautiously elected to use archived canine tissue from dogs with limited cognitive characterization and, therefore, the tissue was not being used for more established pathological studies. While the use of this tissue was sufficient to directly investigate age differences in muscarinic receptor density, the limited level of cognitive data available in these subjects, either in the presence or absence of scopolamine, precludes us from speculating to what extent cognitive status and scopolamine hypersensitivity is linked to muscarinic receptor density changes in dogs. Moreover, future studies examining these potential correlations would likely require more subjects than that used in the current study. Nonetheless, muscarinic receptor density in aged dogs was significantly reduced in all brain regions examined, except the occipital cortex and cerebellum; the aged occipital cortex showed the largest mean reduction, but significance was not reached likely due to the large variability seen in this region. Although the design of the current study prevents us from determining the functional consequences of these alterations, the consistent and significant differences seen across brain regions between young and aged dogs supports the conclusion that muscarinic receptor number is reduced in canine aging.

Another important difference between the two studies was that the aged tissue used in the latter study was from dogs that were slightly older than those used in the scopolamine experiment. Thus, the alterations in muscarinic receptors may reflect a physiological change occurring later in canine aging than the functional differences seen in scopolamine response. On the other hand, the mean differences seen between young and aged dogs in the current study were generally smaller than those reported in humans (Reinikainen et al., 1987; Rinne et al., 1985), suggesting that older dogs may show larger changes than those seen in the current study. Given that initial deposits of amyloid-β begin at approximately 8–9 years of age in dogs (Cotman and Head, 2008), it likely that at least some of the aged subjects in the current study would suffer from some level of amyloid pathology. Interestingly, the pattern of muscarinic receptor loss seen in the present study is consistent with the deposition pattern of amyloid-β previously described in the dog (Head et al., 2000). Specifically, receptor density was not reduced in the cerebellum, which is devoid of plaque formation, and the largest variation occurred in the occipital cortex, which is the region generally affected last by plaque pathology. This potential co-localization could reflect a link between these two pathological events, which has been suggested by others (Hellstrom-Lindahl, 2000). The findings of reduced muscarinic receptors and reduced cholinergic function with age in dogs in combination with previous work indicating dogs can begin to show cognitive deficits as early as 6 years of age (Studzinski et al., 2006) increases the plausibility that age-dependent cholinergic dysfunction contributes, at least in part, to the canine cognitive aging, and this should be more thoroughly investigated in future studies. Nonetheless, this work extends previous findings that aged dogs show pathology and cognitive decline that models the spectrum of normal aging through early Alzheimer's disease and supports the use of the dog to investigate pathological events occurring early in the development of Alzheimer's disease.

The main goals of the present study were to determine if dogs demonstrate age-dependent sensitivity to scopolamine impairment and changes in muscarinic receptor density. The results indicate that, compared to young dogs, aged dogs both are more sensitive to the cognitive disruptive effects of scopolamine on the DNMP task and show decreased muscarinic receptor density. This is the first evidence of physiological alterations in the central cholinergic system of aged dogs, and, in combination with previous data, strongly suggests that cholinergic hypofunction plays a role in age-dependent cognitive decline in dogs, although this was not directly investigated. The current study also warrants extended examination of changes in the canine cholinergic system and potential links with amyloid pathology and cognitive decline. These findings add support for the use of the aged dog as a natural model for examining hypotheses and therapeutics targeting early pathological events in the development of Alzheimer's disease.

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