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Concentrations of platelet α_2-adrenoceptors, lymphocyte muscarinic receptors and blood monoamines in dogs (*Canis familiaris*) affected by canine cognitive dysfunction syndrome

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Abstract

Canine cognitive dysfunction syndrome (CDS) is a neurodegenerative disorder of aged dogs characterized by a progressive decline in cognitive function. In humans and laboratory animals a variety of neurotransmitter abnormalities have been described in patients affected by age-related dementia. Specifically, the regulatory role of the catecholaminergic, serotonergic and cholinergic systems have been outlined. The aim of the present study was to measure blood monoamine levels, platelet α_2-adrenergic receptors and lymphocyte muscarinic receptors in healthy adult and old dogs and in dogs affected by canine cognitive dysfunction. Based on clinical and behavioral examination, 40 dogs were divided into three groups: healthy adults (n=14), old dogs (n=17), and old dogs affected by canine cognitive dysfunction (n=9). A significant reduction in plasma levels of norepinephrine and dopamine was observed both in aged (0.16±0.02 ng/mL; $P<0.01$ and 0.11±02 $P<0.01$ respectively) and CDS dogs (0.14±0.03 ng/mL; $P<0.05$ and 0.10±00.005 $P<0.01$ respectively) compared to adults (0.29±0.04 ng/mL and 0.15±0.02 respectively). No significant differences were observed among groups for α_2-adrenergic receptor concentrations. Canine lymphocytes express two distinct classes of muscarinic receptors, characterized by high (HA) and low (LA) affinity for [^{3}H]-N-methyl-scopolamine. A significant age-dependent decrease in HA muscarinic receptors was observed. However, no differences were found between old (87.65±11.08 sites/cellx10^2) and CDS dogs (90.17±6.75 sites/cellx10^2). As far as LA muscarinic receptors are concerned, CDS dogs showed a significant increase (393.48±63 sites/cellx10^2; $P<0.05$) with respect to adults (188.84±16.50 sites/cellx10^2). Our results suggest that the reduction in HA muscarinic receptor binding sites could be representative of the physiological ageing process, whereas the increase in lymphocyte LA muscarinic receptor levels could be related to the cognitive decline.

Keywords: dog; canine cognitive dysfunction syndrome; catecholamines; serotonin; receptors

Introduction

Ageing represents a complex biological process characterized by a progressive degeneration of tissues and cells with a gradual loss of adaptive capacity. In human aging it is generally associated with a decline in a range of cognitive functions, including learning, memory, visuospatial function, language, information processing speed, and executive function (Adams et al., 2000). In ageing animals a decrease in learning and memory performance can be observed (Milgram et al., 1994; Landsberg and Ruehl, 1997; Adams et al., 2000; Chan et al., 2002). A serious impairment of cognitive processes has to be distinguished from a simple and mild decrease of the psychomotor activity and may be considered as "pathological ageing" (Adams et al., 2000). As in humans, where different levels of ageing and dementia have been classified, various authors have described the so-called "canine cognitive dysfunction syndrome" (CDS) (Landsberg, 2005; Milgram et al., 1994; Cummings et al., 1996a,b). The term canine cognitive dysfunction is used in the veterinary literature to describe a progressive neurodegenerative disorder of aged dogs that is characterized by a gradual decline in cognitive functions such as learning, memory, perception, and awareness (Landsberg, 2005; Milgram et al., 1994; Cummings et al., 1996a,b).

In humans and laboratory animals a variety of neurotransmitter abnormalities have been described in patients affected by age-related dementia (Rehman and Masson, 2001; Buccafusco, 2006; Hirata-Fukae et al., 2008). Nevertheless, these modifications are often associated with specific diseases such as Parkinson's and Alzheimer's diseases (AD) and their involvement in the pathogenesis of age-related cognitive dysfunctions has not been fully elucidated to date (Rehman and Masson, 2001). Ageing has been associated with a decrease in central nervous system dopamine (D) and dopamine receptor (DR) levels and, interestingly, in Alzheimer's disease affected patients a decrease in brain α_2-adrenoceptors (α_2-AR) has been documented (Kalaria et al., 1989; Kalaria and Andorn, 1991; Meana et al., 1992; Sastre et al., 2001).

The serotonergic system seems to be involved in the physiological process of ageing and in the pathogenesis of Alzheimer's disease. In fact, the central nervous system serotonin levels decrease with age, and in AD patients a significant decrease of the neurotransmitter and of some of its metabolites has been observed in the central nervous system and in the cerebrospinal fluid (Baker and Reynolds, 1989; Reinikainen et al., 1990; Tohgi et al., 1992). The change in serotonin levels has been associated with modifications of serotonin receptor concentrations (Lai et al., 2002). It has been suggested that platelets represent a suitable peripheral marker of central serotonergic activity as in AD subjects a significant decrease of central and platelet serotonin concentrations was observed (Kumar et al., 1995; Inestrosa et al., 1993).

According to the "cholinergic hypothesis" of human ageing and Alzheimer's disease, the dysfunction of the cholinergic system contributes to the memory and cognitive decline. The hypothesis has been supported by pharmacological and histological evidence (Bartus et al., 1982; Dunnet and Fibiger, 1993; Araujo et al., 2005). It has been observed that the administration of the muscarinic antagonist scopolamine induces a cognitive impairment in healthy young humans (Drachman and Leavitt, 1974;) and this is increasingly disruptive with advancing age and declining cognitive status (Tariot et al., 1996). Similar findings have been observed in primates, rodents and, more recently, in dogs (Bartus, 2000; Pilcher et al., 1997; Biggan et al., 1996; Araujo et al., 2004). Moreover, in Alzheimer's disease a remarkable decrease of choline acetyltransferase activity in the cerebral cortex and hippocampus has been observed (Davies and Maloney, 1976; Bowen et al., 1997). A correlation between the loss of cortical synapses and cognitive decline was demonstrated as well as a close relationship between this loss and the decrease of high-affinity cholinergic receptors (Withehouse et al., 1982; Nordberg et al., 1992; Hellstrom-Lindahal et al., 1999). Several papers have reported a decrease in muscarinic receptor concentrations in different areas of the central nervous system in both aged humans and rats (Mulugeta et al., 2003; Tayebati et al., 2004; Norbury et al., 2005; Karanth et al., 2007). Furthermore, Tayebati et al. (2001) demonstrated that

AD patients show a decrease in lymphocyte muscarinic receptors3 subtype and an increase in the muscarinic receptors4 subtype (Tayebati et al., 2001).

It has been suggested that canine ageing is similar to some extent to human ageing and that neuropathological changes that occur in dogs are similar to those seen in human aging and in Alzheimer's disease (Studzinski et al., 2005). Aged dogs, like humans, naturally develop amyloid-β deposition and the sequence of the gene encoding for this protein is identical to the human one (Azizeh et al., 2000; Head, 2001). Moreover, dogs and humans share similar patterns of cognitive decline as a function of age (Adams et al., 2000). As a consequence, the dog model is effective in predicting cognitive modifying effects of drugs (Studzinski et al., 2005). Nevertheless, the neurotransmitter systems involved in the pathogenesis of canine cognitive syndrome have not been fully elucidated to date.

Based on this background, the aim of the present study was to measure blood monoamine levels, platelet α_2-adrenergic receptors and lymphocyte muscarinic receptors in healthy adult and old dogs, and in dogs affected by canine cognitive syndrome to identify peripheral markers of the disease and possible targets for specific pharmacological treatments. Previous studies have demonstrated that radioligand binding assays allow the measurement of specific receptor concentrations in different organs and tissues of domestic animals (Badino et al., 2004; Badino et al. 2005).

Material and methods

Animals

Dogs were selected on the basis of history, neurological, cardiology and behavioral examination out of over 124 animals referred to the Teaching Hospital of the Faculty of Veterinary Medicine of the University of Turin. The study was carried out on 40 dogs (26 males and 14 females; weight >

15 Kg). To fulfill the inclusion criteria, the age ranged from 2 to 8 years for the adult group, whereas dogs more than 9 years old were considered as aged. Exclusion criteria were primary organ failure and/or neurological signs. To evaluate the physical condition of dogs, standard clinical examination and laboratory assessments (complete blood count, basic biochemical profile, urinalysis, basic endocrine screening to asses thyroid function) were performed. Moreover, neurological and cardiology examinations have been carried out. In order to diagnose canine cognitive syndrome, the cognitive status of each dog was checked by the veterinary behaviorist filling in a questionnaire (Osella et al., 2007) on the basis of the owners' answers and using cognitive tests aimed to evaluate the short-term visuo-spatial memory (Ghi et al., 2009). Based on clinical and cognitive examinations, dogs were divided into 3 groups: GROUP 1: healthy adults (n=14; age range 2-8 years, mean ± SE 4.3 ± 0.5 years, median 4.5 years); GROUP 2: healthy old dogs (n=17; age range 9-14, mean ± SE 10.5 ± 0.4 years, median 11 years), and GROUP 3: old dogs affected by canine cognitive dysfunction (n=9; age range 9-15 years, mean ± SE 12.0 ± 0.8 years; median 12 years).

All the procedures and sample collections were conducted according to ethical guidelines laid down by the University of Torino (86/609/EEC).

2.3 Blood sample collection and lymphocyte and platelet separation

To obtain suitable biological samples minimizing discomfort to dogs, a method of platelet and lymphocyte separation from a small volume blood aliquot was developed. Twenty ml blood samples were drawn from each dog in tubes containing Na-EDTA. Samples were immediately centrifuged at 240 x *g* for 20 min at room temperature to obtain the platelet rich plasma (PRP) fraction. The harvested PRP fractions were then ultra-centrifuged at 35 000 x *g* for 20 min at 4°C and the resulting pellets were re-suspended in a specific assay buffer (50 mM Tris-HCl, 0.5 mM $MgCl_2$; pH 7.4) and stored at –80°C until the α_2-adrenergic receptor binding assay was run (Pelat et al., 2001).

After removing the platelet rich plasma, the remaining blood was diluted with 2.5 ml of Hank's Balanced Salt Solution (HBSS) and layered into tubes containing 2 ml of Histopaque 1119 (bottom) and 2 ml of Histopaque 1077 (2 ml of blood and 4 ml of Histopaque). Tubes have been centrifuged at 340 x *g* for 30 min at 4°C and the resulting buffy coats, layered between plasma and Histopaque 1077, were centrifuged at 1 660 x *g*, washed twice with HBSS, re-suspended in saline solution (0.9% NaCl; room temperature), counted in a cell counter and stored at –80°C until muscarinic receptor binding assay (Re et al., 1999).

Platelet α_2-adrenergic receptor binding assay

Platelet α_2-adrenergic receptor concentration was assessed using the method described by Pelat et al. (2002), but introducing some minor modifications. Briefly, aliquots of platelet suspension (1.5 mg of protein/ml) were incubated for 30 min at 25°C with increasing concentrations (0.07-10 nM) of [^{3}H]-RX821002, a specific ligand for α_2-adrenergic receptors. Non-specific binding was evaluated by adding 20 μM norepinephrine to the incubating mixture. Incubation was stopped by adding 2 ml of ice-cold buffered saline solution (154 mM NaCl, 50 mM Tris-HCl; pH 7.4) and the incubation mixtures were immediately filtered under vacuum over pre-soaked glass microfiber filters. The filters were then washed with buffered saline and solubilized with 4 ml of scintillation fluid (PicoFluor 40, Perkin Elmer). The radioactivity was measured by the use of a β-counter.

Lymphocyte muscarinic receptor binding assay

Measurements of muscarinic receptor were performed as described by Tayebati et al. (1999) with some minor modifications. Aliquots of lymphocyte suspension (1 x 10^6 cells) were incubated for 1 h at 25°C with increasing concentrations (0.03-4 nM) of [^{3}H]-N-methyl-scopolamine. Non-specific binding was measured in the presence of an excess of atropine (12 μM). At the end of the

incubation, samples were processed as described for the platelet α_2-adrenergic receptor binding assay.

Catecholamine and serotonin blood levels

Epinephrine, norepinephrine and dopamine plasma levels were measured using a commercial RIA kit (TriCat, IBL), whereas serotonin serum concentrations were assessed using a commercial ELISA kit (Serotonin ELISA, IBL) (Badino et al., 2009)

Processing and statistical analysis of data

The equilibrium dissociation constant (K_d) and the number of binding sites (B_{max}), expressed as nM and femtomoles of specifically bound ligand/mg of membrane protein (α_2-adrenergic receptors) or sites/cells x 10^2 (muscarinic receptors), respectively, were calculated by computerized Scatchard analysis (GraphPad Prism). Saturation radioligand binding experiments measure specific radioligand binding at equilibrium at various concentrations of the radioligand and allow the determination of both receptor concentration and radioligand affinity. In the Scatchard plot, the X-axis is the specific binding (B_s) and the Y-axis is specific binding divided by free radioligand (F) concentration (B_s/F) calculated at each concentration of radioligand. It is possible to estimate the B_{max} and K_d from a Scatchard plot (B_{max} is the X intercept; K_d is the negative reciprocal of the slope). The B_{max} value is the highest concentration of radioligand specifically bound to the receptor and represents a measure of receptor concentration. The K_d value is the concentration of radioligand in which a half of receptor is occupied and characterizes the affinity and specificity of the ligand for the receptor (Scatchard, 1949).

Results were expressed as mean values ± SEM (mean standard error). Statistical analysis consisted of multiple comparisons using Tukey-Kramer adjustments to avoid inflation of Type I error due to multiple comparisons (GraphPad Instat).

Results

Platelet α_2-adrenergic receptor concentrations

Scatchard analysis demonstrated the presence of a single class of binding sites saturated by the radioligand concentrations used. Platelet α_2-adrenergic receptor levels and K_d values are shown in Table 1. No significant differences were observed among groups both for α_2-adrenergic receptor concentrations and K_d values.

Lymphocyte muscarinic receptor concentrations

Scatchard analysis allowed identifying in all groups of dogs two distinct receptor binding sites of lymphocyte muscarinic receptors. They were characterized by significant differences in K_d values (Table 2) resulting in high affinity (HA) and low affinity (LA) muscarinic receptors. The muscarinic receptor concentrations are reported in Table 3. Statistical analysis showed significant differences in HA muscarinic receptor levels between GROUP 1 and GROUP 2 dogs ($P<0.01$) and between GROUP 1 and GROUP 3 dogs ($P<0.05$). Significant differences were observed in LA muscarinic receptor concentrations between GROUP 3 and GROUP 1 dogs ($P<0.05$).

Catecholamine and serotonin blood levels

Table 4 summarizes blood monoamine levels (epinephrine, norepinephrine, dopamine and serotonin) in healthy and affected dogs. A significant decrease in norepinephrine and dopamine plasma levels was observed between GROUP 1 and GROUP 2 dogs ($P<0.01$) and between GROUP 1 and GROUP 3 dogs ($P<0.05$ and $P=0.01$, respectively), whereas no significant difference was found between GROUP 2 and GROUP 3 dogs.

Discussion

Data reported in the present study suggest the presence of measurable concentrations of α_2-adrenergic receptors in platelets and muscarinic receptors in lymphocytes of dogs. The binding

method used in our experiment allowed us to identify two distinct classes of lymphocyte muscarinic receptors, characterized by high and low affinity for radioligand. However, the non selective [^{3}H]-N-methyl-scopolamine did not allow us to characterize the different muscarinic receptor subtypes. Currently, five muscarinic receptor subtypes have been identified (M1-M5 receptors). While the odd-numbered receptors (M1, M3, and M5) activate phospholipase C (PLC) leading to phosphatidylinositol 4,5-biphoshonate hydrolysis, agonist activation of even receptors (M2 and M4) leads to adenylyl cyclase inhibition (Ishii and Kurachi, 2006). In the brain of human beings affected by Alzheimer's disease, molecular biology analysis revealed a decrease in M1R and M2R and an increase in M4 subtypes (Flynn et al., 1995), whereas pharmacological assays demonstrated a decrease in M1R-M3R (Rodriguez-Puertas et al., 1997). More recently, Araujo et al., (2011) found that scopolamine significantly impairs performance of aged dogs and that senior dogs show a decreased density of muscarinic receptors in different brain areas. Nevertheless, few studies investigating neurochemical changes and cholinergic dysfunction during canine ageing have been published to date (Woodruff-Pak, 2008). Our data suggest that modifications in lymphocyte muscarinic receptor concentrations reflect those observed in the central nervous system of old dogs. As a consequence, circulating lymphocytes could represent a marker of central cholinergic activity thus allowing *in vivo* studying of cholinergic function in dogs (Bany et al., 1999; Tayebati et al., 1999; Kawashima and Fujii, 2004). In human beings several studies reported that in cognitive dysfunctions, such as Alzheimer's disease or Parkinson's disease, lymphocyte muscarinic receptor concentrations decrease (Ferrero et al., 1991; Rabey et al., 1991). More specifically, AD patients show a decrease in the lymphocyte M3R and an increase in the M4R subtypes (Tayebati et al. 2001). To the best of our knowledge, studies concerning the fluctuation of lymphocyte muscarinic receptors in canine cognitive dysfunction are lacking.

In our study a significant age-dependent decrease in high affinity muscarinic receptors has been observed, however no differences were found between old and CDS dogs. The latter showed a significant increase in low affinity muscarinic receptor concentrations when compared with adult.

These findings suggest that the reduction in high affinity binding sites could be representative of the physiological ageing process, whereas the increase in lymphocyte LA muscarinic receptor levels could be related to the cognitive decline.

In human medicine several studies have been performed to evaluate the involvement of the adrenergic system in Alzheimer's disease. Despite the fact that some results seem to be controversial, some authors observed a decrease in brain α_2-adrenergic receptor concentrations in AD patients (Kalaria et al., 1989; Kalaria and Andorn, 1991; Meana et al., 1992; Sastre et al., 2001). By contrast, Pfeifer et al. (1984) suggested that there is no correlation between age and the number of platelet α_2-adrenergic receptors and between plasma levels of catecholamines and platelet α_2-adrenergic receptors. Present data seem to suggest that neither ageing nor cognitive impairment alter platelet α_2-adrenergic receptor concentrations in dogs.

A variety of neurotransmitter abnormalities have been described in patients affected by age-related dementia. Despite the fact that the most studied neuronal system dysfunction is represented by the cholinergic system, evidences support the involvement of the adrenergic and the serotonergic systems too (Hermann et al., 2004).

The *locus ceruleus* is the major nucleus of origin of noradrenergic neurons in the mammalian brain (Lanari et. al, 2006). This brainstem nucleus is probably involved in sleep, attention, memory, and vigilance. Several studies have demonstrated greater *locus ceruleus* neuron loss in subjects with Alzheimer's disease (Hoogendijk et al., 1999; Storga et al., 1996; Matthews et al., 2002) and similar findings have been observed in dogs with cognitive impairment (Insua et al., 2010). These changes in the *locus ceruleus* may account for a deficiency in the noradrenergic system in the pathogenesis of Alzheimer's disease. Central norepinephrine levels have also been found to have an inverse relationship with cognitive impairment (Matthews et al., 2002). Data obtained in the present study demonstrated a significant reduction in norepinephrine plasma levels in both aged dogs and dogs affected by canine cognitive dysfunction compared to controls. This

finding is only partially in agreement with data reported in the literature, as we did not observe differences between old and CDS dogs.

In the central nervous system serotonin is synthesized and stored in the presynaptic neurons located in nine groups of cell isolated from the pons and midbrain. The *raphe nuclei* represent the major nuclei with both ascending serotonergic fibres projecting to the forebrain and descending fibres that extend to the medulla and spinal cord. Serotonin has been linked to different central nervous system functions such as mood, behavior, sleep cycle, and appetite (Mohammad-Zadeh et al., 2008). Neurochemical and neuropathological disruptions in the serotonergic system such as decreased concentrations of both serotonin and its major metabolite (5-hydroxyindoleacetic acid) in the temporal cortex (Zubenko et al., 1991; Lanctot et al., 2001) have been recognized in the Alzheimer's disease. Studies concerning platelet serotonin concentrations in AD patients yielded controversial results as increased, decreased or unaltered levels have been observed (Kumar et al., 1995; Meszaros et al., 1998; Mimica et al., 2005). However, according to Muck-Seler et al. (2009) differences in peripheral serotonin levels depend on the severity and/or clinical progress of Alzheimer's disease and no significant correlation exists between age and platelet serotonin concentration. As such, the lack of statistically significant differences in blood serotonin levels between adult and CDS dogs observed in our study could be ascribed to the limited number of pathological animals that did not allow a further subdivision according to the severity of cognitive impairment.

To conclude, results of the present study represent the first direct evidence of the involvement of the cholinergic system in canine physiological and pathological ageing. This supports the use of the aged dog as a natural model for examining pathogenetic hypotheses in the development of Alzheimer's disease. Furthermore, our data demonstrate that muscarinic receptors are possible targets for specific drug treatment in dogs affected by cognitive dysfunction.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Table 1. α_2-AR (α_2 adrenergic receptors) concentrations (fmol/mg of protein) and Kd values (nM)

	α_2-AR fmol/mg of protein	K_d nM
Group 1	230 ± 24	0.4 ± 0.05
Group 2	287 ± 28	0.3 ± 0.04
Group 3	290 ± 36	0.4 ± 0.05

Group 1: adult dogs (n=14); Group 2: old dogs (n= 17); Group 3: CDS old dogs (n=9).
Results are expressed as mean values ± SEM.

Table 2. Kd values (nM) of MR (muscarinic receptors)

	K_d MRHA nM	K_d MRLA nM	*P* values
Group 1	0.09 ± 0.02	0.8 ± 0.19	< 0.05
Group 2	0.06 ± 0.01	1.0 ± 0.20	< 0.001
Group 3	0.09 ± 0.02	0.9 ± 0.15	< 0.05

Group 1: adult dogs (n=14); Group 2: old dogs (n= 17); Group 3: CDS old dogs (n=9). MRHA: high affinity binding sites; MRLA: low affinity binding sites. Results are expressed as mean values ± SEM.

Table 3. MR (muscarinic receptors) concentrations (sites/cell x 10^2)

	Group 1 sites/cellx10^2	Group 2 sites/cellx10^2	Group 3 sites/cellx10^2	Group 2 *vs* Group1 *P* values	Group 3 *vs* Group1 *P* values	Group 2*vs* Group 3 *P* values
MRHA	134.81 ± 7.53	87.65± 11.08	90.17 ± 6.75	< 0.01	< 0.05	n.s
MRLA	188.84±16.50	274.38± 27.53	392.48± 63.47	n.s	<0.05	n.s.

Group 1: adult dogs (n=14); Group 2: old dogs (n= 17); Group 3: CDS old dogs (n=9). MRHA: high affinity binding sites; MRLA: low affinity binding sites. Results are expressed as mean values ± SEM.

Table 4. Catecholamines (epinephrine, norepinephrine, and dopamine) and serotonin blood levels (ng/ml).

	Group 1 ng/mL	Group 2 ng/mL	Group 3 ng/mL	Group 2 *vs* Group1 *P* values	Group 3 *vs* Group1 *P* values	Group 3 v*s* Group2 *P* values
Epinephrine	0.25 ± 0.04	0.20 ± 0.06	0.20 ± 0.05	n.s.	n.s.	n.s.
Norepinephrine	0.29 ± 0.04	0.16 ± 0.02	0.14 ± 0.03	< 0.01	< 0.05	n.s.
Dopamine	0.15 ± 0.02	0.11 ± 0.002	0.10 ± 0.005	< 0.01	< 0.01	n.s.
Serotonin	332.17 ± 49.92	300.32 ± 34.14	251.51 ± 44.55	n.s.	n.s.	n.s.

Group 1: adult dogs (n=14); Group 2: old dogs (n= 17); Group 3: CDS old dogs (n=9). Results are expressed as mean values ± SEM.