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AGE-RELATED CHANGES IN THE DOG HYPOTHALAMIC–PITUITARY–ADRENOCORTICAL SYSTEM: NEUROENDOCRINE ACTIVITY AND CORTICOSTEROID RECEPTORS

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Summary—Aging is associated with a progressive dysfunctioning of the hypothalamic–pituitary–adrenocortical (HPA) axis. We have studied the response of the HPA axis to stress and a hormonal (ovine corticotropin releasing factor (o-CRF) challenge in young (1.5–2 years) and aged (>11 years) dogs. Compared to the young dogs, the aged animals displayed an increased basal concentration of both ACTH and cortisol. In addition, in response to an o-CRF challenge (1 μg/kg i.v.) or an electric footshock (1 mA, alternatively on/off for 2 s) or immobilization (45 min) stress, the aged dogs showed significantly larger increments in ACTH and cortisol. Following the challenge test, the young and aged dogs reached their respective basal hormone levels at the same time, except for the o-CRF test. In the latter case, in contrast to the young controls, the aged dogs still showed an increased plasma cortisol level compared to the pre-challenge basal hormone concentration. Concerning the effect of aging on the brain and hypophyseal corticosteroid receptors, a selective decline (minus 50–75%) in mineralocorticoid receptor (MR) was observed in all measured brain regions (dorsal and ventral hippocampus, septum, hypothalamus) and anterior pituitary, whereas no change was found in brain glucocorticoid receptor (GR) number. The GR level in the anterior pituitary was even increased by 70%. In light of the role that MR and GR seem to play in the regulation of the HPA axis, it is concluded that the diminished MR number in the aged dog brain may underly the increased basal hormone levels and the elevated responsiveness of the HPA axis in these animals. The observation that the stress-induced elevations of cortisol and ACTH were not prolonged at senescence suggests that the GR-mediated negative feedback action of glucocorticoids is not altered, which is in line with the unchanged brain GR numbers in the aged dogs.

INTRODUCTION

The aging process of organisms may be regarded as a progressive fall in bodily functions associated with a diminishing ability to maintain homeostasis [1]. Corticosteroids are critical factors in the maintenance of homeostasis and do so via the interaction with two corticosteroid receptor systems in the brain; the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). These receptors have been differentiated on the basis of primary structure [2–6], ligand binding specificity [7, 8], and function [9, 10]. Recently, MR and GR in dog brain and pituitary have been characterized with respect to their steroid binding properties, kinetics and specificity, and their (neuro-)anatomical localization [11]. Canine MR was shown, just like its rodent counterpart, to have *in vitro* highest binding affinity for the physiological glucocorticoids, corticosterone and cortisol, and the mineralocorticoid aldosterone. Interestingly, corticosterone, not being the principal glucocorticoid in the dog, displayed even a 4 times higher affinity for binding MR than cortisol, which is the prime canine glucocorticoid. The dog GR demonstrated highest binding preference for the selective glucocorticoid RU 28362 and did bind corticosterone and cortisol, as compared to their binding to canine MR, with 10- to 100-fold lower affinity [11]. In contrast to the rodent MR, which has a rather stringent localization being virtually limited to the septo-hippocampal complex, the canine MR is found

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much more widespread. Although the septo-hippocampal region is still the most richly endowed brain structure in the dog, also considerable MR levels are found in the hypothalamus and cortical regions. GR is rather evenly distributed in the dog brain and both MR and GR are abundantly present in the anterior and neurointermediate lobe of the canine pituitary [11].

Studies in rats aimed at exploring the function of MR and GR in control of homeostasis have forwarded the notion that these receptors act in a coordinate and antagonistic manner [9, 10]. Corticosterone exerts via limbic MR a tonic action on brain function. This action maintains cellular excitability [12]. Limbic MR-mediated effects are involved in basal control and sets the threshold of responsiveness of the HPA axis [13, 14]. On the behavioral level, corticosterone controls via MR the processing of sensory information and is involved in the development of behavioral strategies [10, M. Oitzl *et al.*, unpublished]. Hypothalamic and hypophyseal GR seem to be involved in the suppression of stress-evoked HPA activity. GR in pyramidal neurons of the hippocampus is involved in suppression of cellular excitability, which is transiently raised by excitatory stimuli [12, 15]. The role of hippocampal GR in neuroendocrine regulation is not clear yet [10, 13, 16], but on the behavioral level, GR-mediated effects promote information storage and recall [16].

At senescence, rat hippocampal MR have been found to be reduced [17–19], in addition to the density of receptor-containing pyramidal neurons [20, 21]. Some studies report a reduced GR number [17], others a reduction in the post-adrenalectomy rise in GR capacity [22], while GR in old Brown Norway rats is not changed [23]. Parallel neuroendocrine experiments have shown a progressive deterioration in the control of HPA activity in the aging rat, resulting sometimes in increased basal levels of corticosterone [19, 24, 25] and in a prolonged adrenocortical secretion following stress [26, 27]. However, impaired adrenocortical function has also been reported at old age, as well as an enhanced stress-responsiveness of pituitary ACTH release [23, 28].

In this study, we report age-related changes in neuroendocrine regulation and central corticosteroid receptors in the dog. As stated before, as in humans, the prime circulating glucocorticoid of the dog is cortisol. In addition, previous studies have demonstrated that, regarding binding characteristics of brain corticosteroid receptors [5, 11] and various aspects of neuroendocrine regulation [29,30], the canine and human HPA system appear to portray similar properties. In that respect, the senescent dog may provide a useful model for studying age-related phenomena in HPA homeostasis in humans.

METHODS

Animals

Twelve young dogs (9 males, 3 females; age 18–24 months) and 10 aged dogs [6 males, 4 females; age over 11 years (mean 13 years)] were used; 6 young and 6 old dogs were beagles and the others were mongrels. The animals were individually housed in indoor cages and they were fed a commercial pellet food, and water was supplied *ad libitum*.

Neuroendocrine challenge tests

The tests were performed with intervals of 7–10 days and all tests were started at 9.00 a.m. Blood samples were drawn by jugular vein puncture and collected in ice-chilled EDTA-coated tubes. Within 30 min after blood sampling, plasma was isolated and immediately frozen at −20°C. Cortisol was measured by a radioimmuno assay (RIA) procedure [31]. The following challenge tests were performed:

(1) CRH-test. Ovine CRF (o-CRF; 1 μg/kg body wt) was administered intravenously (i.v.) and blood samples were drawn at various time intervals between 0 and 180 min following the i.v. injection.

(2) Electric footshock stress. The dogs were placed in left lateral recumbancy and electric shocks (1 mA, alternatively on/off for 2 s) were given for 1 min via 2 subcutaneous needles placed 2 cm apart in the dorsal carpal region of one forelimb with an earth connection at the other forelimb. Blood samples were taken at various time intervals between 0 and 150 min after starting the electric shocks.

(3) Immobilization stress. Dogs were immobilized in dorsal recumbancy by fixation of their extremities for 45 min. Blood samples were drawn immediately before the onset of immobilization and at 15 min intervals thereafter until $T = 195$ min. Apart from the aforementioned blood samples, blood samples were also drawn at 30 and 15 min, and immediately before the start of each challenge test to obtain estimates of the basal concentrations of cortisol and ACTH.

Adrenalectomy and tissue dissection

In order to perform corticosteroid receptor assays on tissue samples of the dogs used in the neuroendocrine challenge tests, the animals were bilaterally adrenalectomized in order to deplete the body of endogenous corticosteroids. Anesthesia was initiated with i.v. thiopental and maintained with halothane, N_2O and O_2 inhalation anesthesia. Following ADX the dogs were given 0.9–1.8% NaCl via an indwelling catheter in the jugular vein to maintain Na^+ homeostasis until the time of sacrifice. The dogs were sacrificed 1 day after ADX by anesthesia with pentobarbital followed by perfusion of the head with ice-cold 0.9% NaCl via the carotid arteries. Brain perfusion removed plasma cortisol binding globulin (CBG), which interferes with the *in vitro* [^{3}H]cortisol binding assay. Blood just collected before anesthesia was used to determine endogenous corticosteroid levels. After perfusion, the skull was opened and the brain and pituitary removed. The pituitary was disconnected from the brain and the anterior pituitary (AP) was dissected. The brain was rapidly dissected into neuroanatomically defined tissues with the aid of the atlas of Dua-Sharma *et al.* [32]. These tissues were the dorsal and ventral hippocampus, septum and hypothalamus. The dissected tissues were frozen immediately on dry ice and stored at −70°C until cytosol receptor assay. The protocols for the neuroendocrine challenge tests, surgery and euthanasia were approved by the Institutional Committee on Animal Care and Use of the Veterinary Faculty, State University of Utrecht.

Tissue homogenization and cytosol preparation

Brain or pituitary tissue samples of individual dogs (of both sexes) were homogenized (10 ml/g; 10 strokes at 500 rpm) in ice-cold 5 mM Tris–HCl (pH 7.4) containing 5% glycerol, 10 mM sodium molybdate, 1 mM EDTA and 1 mM β-mercaptoethanol using a glass homogenizer with a Teflon pestle milled at a clearance of 0.250 mm on the radius. The homogenate was centrifuged at 100,000 *g* for 60 min at 0–2°C to obtain cytosol (i.e. supernatant fraction). Specific binding of ^{3}H-labeled ligands in cytosol preparations was assessed with an *in vitro* receptor assay.

Corticosteroid receptor assay

Aliquots of cytosol (100 μl) were incubated with ^{3}H-labeled steroids either over a concentration range of 0.1–15 nM (brain tissues) or at one concentration (15 nM; >96% saturation; AP) in duplicate (total incubation volume 150 μl). Total binding to MR was determined with [^{3}H]cortisol or [^{3}H]aldosterone in the presence of a 50-fold excess of the highly selective glucocorticoid agonist RU 28362. Nonspecific binding was assessed in parallel incubations containing a 1000-fold excess of the appropriate unlabeled steroid (cortisol or aldosterone). However, the remaining specific [^{3}H]cortisol binding after inclusion of RU 28362 represented not only binding to MR, but also to remnants of CBG due to blood contamination. CBG was determined by subtracting the nonspecific binding from the binding of [^{3}H]cortisol obtained in the presence of a 500-fold excess of dexamethasone. Hence, the MR binding data were corrected for the amount of CBG present in the cytosol. The CBG contamination usually did not exceed 3 fmol/mg protein. The dog GR was determined with [^{3}H]RU 28362. Nonspecific binding was measured in parallel incubations containing a 1000-fold excess of unlabeled RU 28362. Binding equilibrium was reached after an overnight incubation of 20–24 h at 0°C. Separation of bound and free ^{3}H-labeled steroid occurred by LH-20 gel filtration and measurement of protein was done according to the method of Lowry *et al.* [33] with BSA as the standard. Binding data were expressed as fmol/mg protein and the specific binding was calculated. The apparent maximal binding capacity (B_{max}) and apparent affinity (K_d) of MR and GR were calculated by Woolf and Scatchard analysis, respectively.

RESULTS

Neuroendocrine challenge tests

Figure 1 shows plasma cortisol levels at three distinct phases of the neuroendocrine challenge tests: pre-challenge basal levels, peak concentrations and the "recovery" phase (120 min post-challenge). From Fig. 1(A–C) it is evident that, compared with the young dogs, aged dogs display elevated levels of cortisol (Student's t-test, $P < 0.05$). Also, basal ACTH levels are increased (not shown). Moreover, upon administration of o-CRF or application of stress, the aged animals showed a significantly larger responsiveness in cortisol [(Student's t-test, $P < 0.05$); Fig. 1] and ACTH (not shown). Peak levels of cortisol as a result of o-CRF administration and electric footshock

and immobilization stress were attained at different times; after administration of o-CRF at 30 min, and after electric footshock stress and immobilization stress at 10 and 45 min, respectively. At 120 min after o-CRF administration ["recovery", Fig. 1(A)], the young dogs reached basal pre-CRF concentrations, while, in contrast, plasma cortisol concentrations in the aged animals still were above their respective basal level. In the recovery phase after either stress the young as well as the old dogs had reached their respective pre-stress (basal) levels again [Fig. 1(B, C)]. No sex differences were apparent in any of the tests.

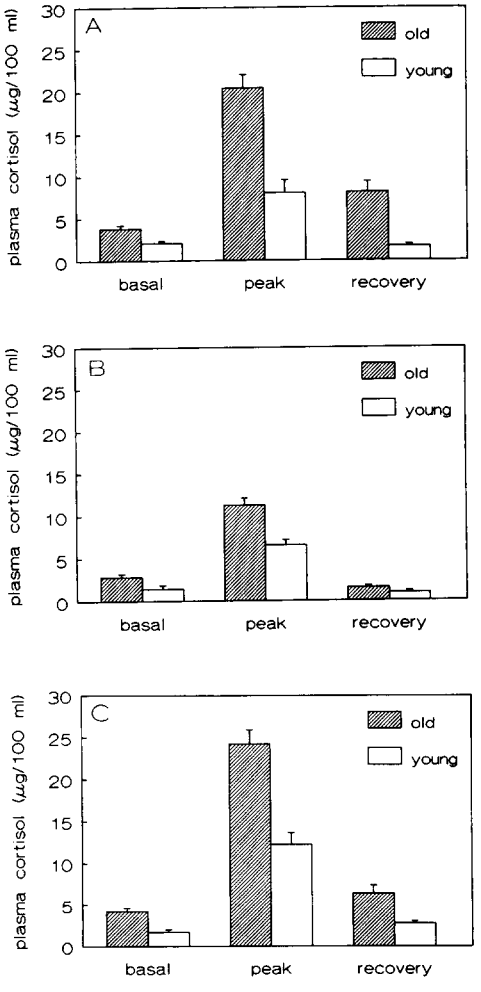

Fig. 1. Effect of o-CRF (1 μg/kg i.v.; A), electric footshock (B) and immobilization stress (C) on plasma cortisol levels in young and aged dogs. Plasma cortisol concentrations (mean ± SEM) are expressed as μg/100 ml. For details on the electric footshock and immobilization procedure, see Methods. "Basal" refers to pre-challenge plasma cortisol concentrations in young and aged dogs, "peak" and "recovery" refer to the post-challenge maximal response in plasma cortisol and to the steroid level 120 min after the onset of the challenge, respectively (see also Results).

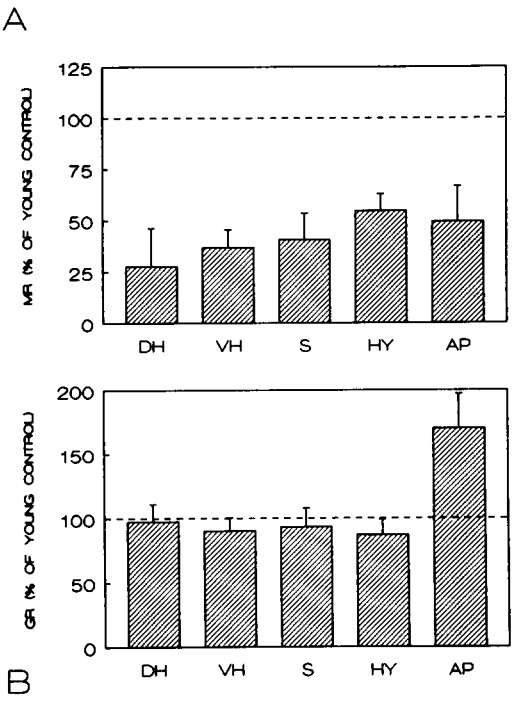

Fig. 2. Effect of aging on MR and GR concentration in dog brain and pituitary. For experimental details, see Methods and Results. Data are expressed as a percentage (%, mean ± SEM) of the number of receptors found in young animals. *Abbreviations*: DH = dorsal hippocampus, VH = ventral hippocampus, S = septum, HY = hypothalamus, AP = anterior pituitary.

Age-related corticosteroid receptor changes

Figure 2 shows the corticosteroid receptor levels in brain and pituitary of aged dogs, expressed as a percentage of the concentrations found in young animals. As shown in Fig. 2(A), MR concentration is markedly decreased in all tissues examined (minus 50–75%; Student's t-test, $P < 0.05$), with the highest decrements in the extrahypothalamic limbic brain regions, septum and dorsal and ventral hippocampus (minus 65–75%). In contrast, GR levels were found not to be decreased in the aged canine brain, while GR levels in the AP were increased by 70% (Student's t-test, $P < 0.05$).

DISCUSSION

In the present study, we characterized the neuroendocrine response of young and aged dogs after immobilization, electric footshock, and in response to a hormonal (o-CRF) challenge. We find hyperresponsiveness of the LHPA axis during aging as measured by elevated peak levels of ACTH (not shown) and cortisol. In addition, the aged dogs showed a

significant increase in basal circulating concentrations of ACTH and cortisol. Measurement of corticosteroid receptor binding properties in various parts of the canine brain revealed that in the aged dog brain a selective decline in MR was apparent in all measured brain regions. Among these brain regions highest decrements were found in the extrahypothalamic limbic areas. MR levels in the AP were also decreased. In the case of brain GR, no such age-associated changes were measured, while anterior hypopheseal levels of GR were markedly elevated in the aged dog.

In this study, aged dogs displayed hypercortisolemia not only under basal conditions but also as a result of a stressful or hormonal challenges, In the aged rat, some authors have found increases in basal and stress induced levels of plasma corticosterone [19, 24, 25], whereas others have found no such changes [28,34–38]. The use of different rat strains as well as differences and severity in the challenges may underly the variability in the outcome of the various studies. In addition, a marked sex difference in plasma corticosterone level has been reported in rats [36, 39]. In this study, no sex difference was observed in either young or aged dogs. Recently, in young and aged male Brown Norway rats, van Eekelen *et al.* [23] studied the stress-responsiveness of the HPA axis in relation to the properties of the brain and hypophyseal corticosteroid receptors. The aged rats displayed increased stress-responsiveness of the pituitary ACTH release, while plasma corticosterone levels were not altered under resting conditions or after stress [23]. In the present study, the pattern of neuroendocrine responses in cortisol in the young and aged dogs largely paralleled the changes in ACTH.

The present study shows for the first time that, in the aged dog, brain and hypophyseal MR are largely decreased, while GR in brain is not affected and is even upregulated in the AP. No apparent differences in age-related effects on corticosteroid receptors were found among different dog strains [J. M. H. M. Reul *et al.*, unpublished]. In aging studies using the rat as an experimental model, the situation is more complex. In the aged rat brain and pituitary, depending on the rat strain used and the brain tissue under study, some studies report a decrement in both MR and GR, while others show a rather specific decrement in MR with no change in the levels of GR. An example of the first-mentioned category is a study by Reul *et al.* [17], in which a 52% reduction in MR and a 28% decrease in GR was found in the hippocampus of 28- to 31-month-old male Wistar rats. Also, in other brain regions (hypothalamus, septum, caudate nucleus) these authors found a 25–30% reduction in GR [J. M. H. M. Reul and E. R. de Kloet, unpublished]. Van Eekelen *et al.* [23] found a selective decrease in hippocampal MR capacity in aged male Brown Norway rats, while hippocampal GR was not affected. GR concentration was found to be reduced in the hypothalamus and AP of these animals [23]. Similar age-related changes were observed in 17.5-month-old Fisher 344 rats, in which only a selective decline in hippocampal MR was found [28]. In aged Long Evans rats, a reduction in hippocampal GR was apparent, but no change in GR was detectable in the hypothalamus or the AP [40]; MR was not measured in this study. Lastly, Eldridge *et al.* [22] found no changes in hippocampal GR in 24-h adrenalectomized aged rats, but a decrement in GR became apparent when receptors were measured 8–10 days after ADX. These authors concluded that hippocampal GR in the aged rat apparently is impaired in the capacity to upregulate its concentration in the absence of adrenocortical secretion, which may indicate a decreased plasticity of this receptor at senescence [22]. Taken together, hippocampal MR concentration is found to be decreased in all studies, while GR numbers in the tissues are either decreased or not altered.

It has been hypothesized that corticosterone and cortisol exert a dual control on the activity of the HPA system, being a tonic influence via the limbic MR and a feedback action (post-stress) via GR [8–10]. Since MR and GR presumably are colocalized in the pyramidal neurons of the hippocampal formation and other neurons in limbic structures, it is thought that the balance of MR and GR functioning may be important for the homeostasis of the HPA axis. Recent studies by Dallman's and de Kloet's group have shown that it is the limbic MR, which is mostly involved in the regulation of basal ACTH secretion [13, 14]. The present study shows that limbic MR levels are diminished in the aged dog brain, which may explain the elevated basal concentrations of plasma ACTH and cortisol and the increased stress-responsiveness of these animals. The stress-induced elevations of cortisol and ACTH were

not prolonged, which suggests that GR-mediated negative feedback action of glucocorticoids is not altered. This observation is in line with the unchanged brain GR, as reported in this study. In this context, the significance of the increased capacity of GR in the AP is at present unknown.

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