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Immunohistochemical analysis of the hypothalamic-pituitary-adrenal axis in dogs: Sex-linked and seasonal variation

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ABSTRACT

This study evaluated sexual dimorphism and seasonal variations in corticotrophs and adrenal zona fasciculata in dogs, as well as the expression of oestrogen receptor alpha (ERα). An immunohistochemical analysis was conducted in pituitaries for ACTH and in adrenal glands for ERα and for the melanocortin-2-receptor (MC2R) in winter and summer. Double immunofluorescence was performed to identify ERα in corticotrophs. Females had a greater proportion of corticotrophs per field ($p < 0.01$), with a greater cellular area and optical density ($p < 0.001$) than males. Optical density of corticotrophs was greater in winter for both sexes ($p < 0.001$). In zona fasciculata, ERα and MC2R expression was greater in females ($p < 0.001$) and was greater in winter ($p < 0.001$). ERα was identified in corticotrophs. This study is the first to demonstrate ERα expression in corticotrophs and the adrenal cortex in dogs, providing evidence for sexual dimorphism and seasonal variations.

1. Introduction

Functional sexual dimorphism in the hypothalamus-pituitary-adrenal (HPA) axis has been described in several species, including sheep, rats and, monkeys (van Lier et al., 2014). The activation of this axis is suggested to be sex-dependent, and in rat models, females are considered to have a greater stress response than males (Iwasaki-Sekino et al., 2009; Larkin et al., 2010). Sex-associated differences in HPA axis response have also been observed under basal conditions in rats and humans (Viau et al., 2005, Goel et al., 2014; Handa and Weiser, 2014). Pessina et al. (2009) reported differences according to sex in the cortisol response to adrenocorticotropic hormone (ACTH) stimulation and dexamethasone inhibition in healthy dogs, being this response greater in female dogs than in male dogs. Also, recently our group has reported variations in ACTH and cortisol plasma concentrations during the oestrous cycle in the dog (Gallelli et al. 2015). Rhodes and Rubin (1999) postulated that in mammals the differences in HPA axis function (hormone production) between sexes could be a result of morphological dimorphism (cell size, shape or number). The morphological dimorphism of corticotroph cells has been described in vizcachas (*Lagostomus maximus*), where female vizcachas have smaller but more numerous corticotrophs than males (Filippa and Mohamed, 2006). Also, in a study on the American mink (*Mustela vison*), Vidal et al. (1995) reported a considerable increase in the size of the area of corticotroph cells coinciding with the onset of puberty in females but not males, suggesting that this process may be affected by gonadal steroids. Although there are numerous reports detailing the effects of oestradiol on the HPA axis, the mechanisms of the hormone's action on this axis have not been completely elucidated (Handa and Weiser, 2014). Mitchner et al. (1998) identified oestrogen receptor alpha (ERα) in the corticotroph cells of the anterior lobe and in melanotrophs of *pars* intermedia in rats. Similarly, this receptor was found in the adrenal cortex of rats and sheep (Cutler et al., 1978; van Lier et al., 2003). Through ERα, oestradiol could stimulate adrenal steroidogenesis or affect adrenal sensitivity to ACTH as it has been reported in rats and sheep (Figueiredo et al., 2007; van Lier et al., 2014).

Differences in the cellular area and distribution of corticotroph cells in the pituitary gland have been described in certain species in different photoperiods, suggesting that these characteristics may be sensitive to seasonal variation (Hira et al., 2001; Filippa and Mohamed, 2006). This result is similar to changes described in the adrenal gland of vizcacha in which the nuclear volume of the adrenal cortex also shows seasonal variation (Ribes et al., 1999). Seasonal differences in ACTH and cortisol plasma concentration have also been reported in different species (Ingram et al., 1999; Romero, 2002; Cordero et al., 2012; Gallelli et al., 2015).

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To our knowledge, no studies have described the sexual dimorphism in the HPA axis morphology in dogs or seasonally associated changes. Thus, the aim of this study was to analyse whether morphological differences in corticotrophs occur between male and female dogs through different seasons in a year, and determine if the ERα is expressed in these cells. Also, this study aimed to evaluate whether ERα is found in the adrenal zona fasciculata and if its expression and that of the ACTH receptor (melanocortin-2-receptor, MC2R) varied by sex and season. These results would provide new information about HPA axis' morphology and receptor expression and would broaden the knowledge of its -characteristics. Taking into consideration the model proposed by Rhodes and Rubin (1999) this data could open up a way to studying the relationship between morphological and functional sexual dimorphism in the dog.

2. Materials and methods

2.1. Population under study

This study was approved by the ethics committee of the School of Veterinary Sciences and grant committee (CICUAL, UBACyT V006 and 20020100100246) of the University of Buenos Aires. The study was conducted in the city of Buenos Aires, Argentina (latitude: 34°30'S, longitude: 58°26'W, altitude 25 m.a.s.l; December 21st is the summer solstice and June 21st is the winter solstice). During the summer, there are more than 12 h of light per day (average 14 h, 15 min) and the mean temperature is 22.3 °C. During the winter, there are fewer than 12 h of light per day (average 10 h 4 min) and the mean temperature is 12.5 °C.

The pituitary and adrenal glands were obtained from dogs that were euthanized for humanitarian motives following recommendation by a veterinarian (e.g., medullar trauma). All animals underwent clinical evaluation before euthanasia. At that moment dogs were not in pain (assessed by Glasgow pain scale). Dogs suspected of suffering from infectious diseases or diseases that may affect the HPA axis (oncological diseases) were excluded from the study as were dogs that received glucocorticoid treatment prior to euthanasia. Dogs diagnosed with the aforementioned diseases during necropsy were also excluded from the study.

In summer, 7 female and 7 male dogs, all non-neutered, were included in the study, while in winter 7 female and 6 male dogs, all non-neutered, were considered. During the summer, samples were obtained from 5 mixed and 2 Labrador Retriever females (mean age: 8 years; mean weight: 29 kg) as well as 5 mixed and 2 Boxer males (mean age: 8 years; mean weight: 29 kg). During the winter, samples were obtained from 4 mixed and 3 Golden Retriever females (mean age: 8 years; mean weight: 31 kg) as well as 4 mixed and 2 Doberman males (mean age: 7 years; mean weight: 32 kg).

2.2. Immunohistochemical analysis

2.2.1. Immunostaining of ACTH in pituitary gland

Pituitary glands were fixed in 4% buffered formol and embedded in paraffin. Sagittal sections (5 μm thick) were mounted on silanised slides and subsequently rehydrated by carrying them through xylol and graduated alcohols, and finally through phosphate buffered saline (PBS, 0.01 M, pH 7.4). Sections were incubated for 20 min in a 3% H_20_2 solution in distilled water to inhibit endogenous peroxidase activity. After washing with PBS, non-specific binding sites were blocked by incubating in normal goat serum for 10 min ("Immunoperoxidase secondary detection system," DAB 150, Millipore, Temecula, CA, USA). Sections were washed with PBS and then, in order to identify corticotrophs, they were incubated in a humidified chamber at room temperature for 2 h with the primary anti-ACTH antibody (mouse monoclonal, sc-52,980, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:400 dilution in PBS. This antibody has been raised against 1–24 amino acids, which are common to all mammals (Mol and Meij, 2008). After washing in PBS, the "Immunoperoxidase secondary detection system" kit was used (DAB 150, Millipore, Temecula, CA, USA). Using this system, sections were incubated for 10 min with the anti-mouse biotinylated antibody. After washing with PBS, sections were then incubated for 10 min with Streptavidin-conjugated horseradish peroxidase. After a final wash with PBS, HRP-bound sites were developed with the chromogenic reagent 3,3′ diaminobenzidine (DAB). All sections were processed during the same session and had the same development time. Sections were lightly stained with haematoxylin, dehydrated and mounted. Negative controls were performed as above, replacing the primary antibody with PBS.

2.2.2. Double immunofluorescence staining of ACTH and ERα in pituitary gland

ACTH and ERα colocalisation was evaluated. The pituitary gland from each animal was fixed, embedded in paraffin, sectioned and rehydrated as described previously for "*Immunostaining of ACTH in pituitary gland*". REα antigen retrieval was performed. With this purpose, sections were placed in citrate buffer (0.01 M, pH = 6.0) and heated in the microwave (900 W) for 10 min. Sections were allowed to cool at room temperature and then washed with PBS. Non-specific binding sites were blocked with normal goat serum ("Immunoperoxidase secondary detection system," DAB 150, Millipore, CA, USA) for 10 min. Sections were incubated overnight at room temperature in a humidified chamber with the anti-ERα primary antibody (mouse monoclonal, sc-787, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:25 in PBS. The sections were washed with PBS and incubated for 1 h with the anti-mouse biotinylated antibody (E0354, DAKO) diluted 1:125 in PBS, which was followed by a 1 h incubation with streptavidin-conjugated FITC (SA 100-02, Molecular Probes, Invitrogen) diluted 1:100 in PBS. After washing with PBS, the sections were incubated overnight in a humidified chamber at room temperature with the anti-ACTH primary antibody (rabbit-anti human ACTH, NIDDK; raised against 1–24 amino acids) (it was chosen in order to use an antibody raised in a different species than the ERα antibody) diluted 1:100 in PBS. The sections were washed with PBS and incubated for 1 h with anti-rabbit rhodamine antibody (AP132R; Chemicon International) diluted 1:100 in PBS. Finally, the sections were mounted in PBS-glycerol (1:1). Negative controls were performed as above, replacing the primary antibody with PBS.

2.2.3. Immunostaining for ERα and MC2R in adrenal glands

The adrenal glands of all dogs were fixed, embedded in paraffin, sectioned and rehydrated using the protocol previously described for the immunostaining in pituitary glands. Antigen retrieval was performed on sections used for ERα immunostaining as described for the pituitaries. All of the sections were then incubated with 3% H_20_2 in distilled water for 20 min before being washed with PBS. Non-specific binding sites were blocked with normal horse serum for 30 min (Vectastain ABC-Kit, Elite, PK-6102, Burlingame, CA, USA). Sections were incubated in a humidified chamber for 2 h with either a 1:100 PBS:anti-MC2R primary antibody dilution (rabbit polyclonal; sc-13107, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or with a 1:25 dilution of the anti-ERα primary antibody (mouse monoclonal, sc-787, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS (Ithurralde et al., 2013). After being washed with PBS, sections were incubated for 1 h with a 1:200 dilution of the biotinylated anti-rabbit antibody (BA-1000, Vector, Burlingame, CA, USA) or the biotinylated anti-mouse antibody (BA-200, Vector, Burlingame, CA, USA) as appropriate for each section based on the previously applied primary antibody. Sections were washed with PBS before use of the "Vectastain Elite ABC Kit" detection system (Vector, Burlingame, CA, USA). The following steps were completed as described for sections of the pituitary gland. In all cases, negative controls were conducted via replacement of the primary antibody with PBS.