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Expression and Functional Analysis of Dopamine Receptor Subtype 2 and Somatostatin Receptor Subtypes in Canine Cushing's Disease

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Cushing's disease (CD) is a severe disorder characterized by chronic hypercortisolism due to an ACTH-secreting pituitary adenoma. Transsphenoidal adenomectomy is the treatment of choice in humans with CD, but recurrences occur frequently. Finding an effective and safe medical treatment for CD may improve long-term clinical outcome. The recent demonstration of expression of somatostatin receptor subtypes (mainly sst_5) and dopamine receptor subtype 2 (D_2) in human corticotroph adenomas offers the possibility for medical treatment of CD with novel somatostatin analogs and dopamine agonists. Investigation of the effects of these drugs is hampered by the low incidence of CD in humans. Interestingly, CD is a frequent disorder in dogs with striking clinical similarities with CD in humans. Therefore, we investigated the expression and functional role of D_2 and somatostatin receptors in corticotroph adenoma cells from 13 dogs with active CD that underwent therapeutic hypophysectomy and normal anterior pituitary cells from five dogs. Quantitative RT-PCR and immunohistochemistry revealed that both in CD and normal anterior pituitary, sst_2 was the predominant receptor subtype expressed, whereas D_2 was modestly expressed and sst_5 was expressed only at very low levels. In primary cultures of canine adenomas (n = 7), the sst_2-preferring agonist octreotide also showed the strongest ACTH-suppressive effects. In conclusion, canine corticotroph adenomas provide an interesting model to study CD, but differences in somatostatin and dopamine receptor expression between humans and dogs should be taken into account when using dogs with CD as a model to evaluate efficacy of novel somatostatin analogs and dopamine agonists for human CD. (*Endocrinology* 149: 4357–4366, 2008)

CUSHING'S DISEASE (CD) is a severe endocrinological disorder due to an ACTH-producing pituitary adenoma. The resulting chronic hypercortisolism causes significant morbidity and, if left untreated, mortality in these patients (1). Primary treatment of CD is transsphenoidal selective adenomectomy (2) but results in long-term cure in only 50–80% of patients (3). Secondary treatments such as radiotherapy or bilateral adrenalectomy are generally effective but can cause permanent hypopituitarism or the necessity of life-long adrenal hormone replacement therapy, respectively.

For that reason, finding an effective and safe medical therapy for human CD can be of great importance for those CD patients that are not cured by neurosurgery alone. Various drugs have been used in patients with CD, but most of them have not been efficacious in long-term treatment or are associated with an unfavorable safety profile (4). Novel drug targets have been identified, however, as it was found that the somatostatin (SS) receptor subtype 5 (sst_5) and the dopamine (DA) receptor subtype 2 (D_2) are expressed in the majority of human corticotroph adenomas (5–7). Compounds that target these receptor subtypes, such as the multiligand SS analog with high sst_5 affinity pasireotide (PAS, or SOM230) and the D_2-agonist cabergoline (CAB), have already shown in some *in vitro* and *in vivo* studies to decrease ACTH release by corticotroph adenoma cells and thus lower cortisol levels (5, 6, 8).

For the development of new medical therapies in human CD, research on primary corticotroph adenoma tissue is crucial. The efficacy of new compounds in CD can only be genuinely tested in the cell type they are primarily directed at, *i.e.* the human corticotroph cell. This tissue can be obtained only at the time of transsphenoidal adenomectomy in a CD patient. Due to the low incidence of CD of approximately 1.2–2.4 cases per million per year (9, 10) and the fact that 80–90% of these cases are due to microadenomas with a diameter of less than 10 mm (11, 12), there is a severe shortage of human corticotroph tissue, which limits research options in human CD. For that reason, finding ways to increase the availability of primary corticotroph adenoma tissue is a major challenge in this research field.

In contrast to the situation in humans, CD is a frequent endocrinological disorder in dogs, with an estimated incidence of one to two cases per 1000 per year (13–16). Canine CD, also referred to as pituitary-dependent hyperadreno-

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Abbreviations: CAB, Cabergoline; D_2, dopamine receptor subtype 2; DA, dopamine; DEX, dexamethasone; IHC, immunohistochemistry; NeuroD1, neurogenic differentiation factor D1; OCT, octreotide; PAS (or SOM230), pasireotide; P/B, pituitary height-to-brain area ratio; PDH, pituitary-dependent hyperadrenocorticism; POMC, proopiomelanocortin; qPCR, quantitative PCR; SS, somatostatin; sst_2, somatostatin receptor subtype 2; sst_5, somatostatin receptor subtype 5; UCCR, urinary corticoid-to-creatinine ratio.

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corticism (PDH), has a remarkably similar pathophysiology and clinical presentation as CD in humans and can hence be regarded as a spontaneous animal model for human CD (17). In dogs with CD, true microadenomas are rare, and pituitaries are frequently enlarged (18). Medical treatment of dogs with CD involves the use of adrenolytic drugs such as mitotane or an inhibitor of steroidogenesis such as trilostane (19, 20). In The Netherlands, hypophysectomy has been performed in dogs with CD since 1993 and has proven to be a safe and effective treatment (18, 21, 22). The procedure consists of a complete hypophysectomy via a transsphenoidal approach as is described in detail elsewhere (22).

Given the high incidence of CD in dogs, the high degree of similarity with human CD and the availability of corticotroph adenoma tissue obtained at hypophysectomy, we hypothesized that evaluation of the efficacy of new compounds for treatment of human CD may be tested first in canine corticotroph adenoma tissue. Therefore, our main study aim was to characterize these canine corticotroph adenomas for the expression and functional role of those receptor subtypes that are of primary interest in the research of human CD, sst_2, sst_5, and D_2, and to compare these results with the current knowledge on human corticotroph adenomas.

Materials and Methods

Study population

Thirteen dogs [five females (four spayed) and eight males (three castrated)] with CD (*i.e.* PDH) from various breeds were included in the study (Table 1). The median age was 8 yr (range, 5–14 yr), and the median body weight was 23.2 kg (range, 6.7–48.0 kg). Hypercortisolism was diagnosed by clinical signs, routine laboratory investigation, and determination of the urinary corticoid-to-creatinine ratio (UCCR) in two consecutive morning urine samples as described previously (23–26). The mean UCCR was 116.7×10^{-6} (range, 26.5–302.5×10^{-6}; normal, $<10 \times 10^{-6}$) (18). After collection of the second urine sample, three oral doses of 0.1 mg dexamethasone/kg body weight were administered at 8-h intervals, and the next morning, a third urine sample was collected (high-dose dexamethasone suppression test). In 10 dogs, the UCCR in the third sample was less than 50% of the mean in the first two samples, and PDH was diagnosed (18). In two cases with less than 50% suppression, dexamethasone-resistant PDH was confirmed by measurements of plasma ACTH concentrations and further supported by visualization of the adrenals by ultrasonography and pituitary imaging (27–30). Computed tomography of the pituitary gland revealed pituitary enlargement in each case, except one (C8), with a mean pituitary height-to-brain area ratio (P/B) of 0.58 (range, 0.30–1.00; pituitary enlarged when P/B $> 0.31 \times 10^{-2}$ mm^{-1}) (31). Plasma cortisol, ACTH, and α-MSH concentrations were determined with assays that have been described previously (32). Preoperative mean (+ range) plasma values were α-MSH 27.8 (<5–224) pg/ml, cortisol 196.9 (61–414) nmol/liter, and ACTH 21.5 (9.3–41.8) pmol/liter (see Table 1 for reference values). Microsurgical transsphenoidal hypophysectomy was performed as published previously (22).

Unaffected pituitary tissue was obtained from five Beagle dogs, which had been euthanized for reasons unrelated to the present study and for which approval was obtained from the Ethical Committee of Utrecht University, The Netherlands. The pituitary gland was collected within 10 min after euthanasia. The anterior pituitary was separated from the neurointermediate lobe, and the anterior pituitary was processed for analysis.

Surgical tissue and cell isolation

During transsphenoidal hypophysectomy, pituitary adenomatous tissue was identified macroscopically by the veterinary surgeon and resected. A representative part of the adenoma was fixated in 4% buffered paraformaldehyde and sent for histopathology for hematoxylin and eosin staining and immunohistochemistry (IHC) to evaluate ACTH, α-MSH, and GH expression (33). The surplus adenomatous tissue was immediately placed in a prechilled (4 C) solution of MEM with Earle's salts, supplemented with 10% fetal calf serum, L-glutamine (2 mmol/liter), penicillin (10^5 U/liter), and fungizone (0.25 mg/liter). Media and supplements were obtained from Invitrogen (Breda, The Netherlands). Upon arrival in the laboratory, the adenoma tissue was further divided into two parts; one part was snap-frozen on dry ice and stored at −80 C for quantitative PCR (qPCR) studies, and the other part was kept overnight at 4 C in MEM. Next day, the latter adenoma part was washed in Hanks' balanced salt solution/human serum albumin 1%, dispersed with 10^3 U/liter dispase (Roche, Almere, The Netherlands) plus 2 mg/ml collagenase (Sigma Aldrich, Zwijndrecht, The Netherlands) at 37 C for 1 h, and resuspended in MEM complete culture medium. Viable pituitary cells were counted in a standard hematocytometer.

Cell distribution and culture

The average yield per tumor in terms of viable canine pituitary cells was 2.4×10^6 cells (range, 0.5–11.0×10^6). Of these cells, 0.2×10^6 were used for qPCR studies and 0.1×10^6 for the preparation of cytospins for IHC (see below). The remainder of the cells was cultured in 48-well plates (Corning, Cambridge, MA) at a density of 10,000 cells per well for 4–6 d at 37 C in a humidified incubator in 5% CO_2. At that time, culture media were refreshed and incubations were started with the different DA agonists and SS analogs for 4–72 h. Both basal and CRH-induced ACTH release was studied. At the end of the incubation period, media were collected and stored at −80 C for hormone analysis after addition of aprotinin (4×10^5 IU/ml medium; Bayer, Mijdrecht, The Netherlands) to prevent ACTH degradation. All experimental conditions were performed in quadruplicate.

Hormone analysis in vitro

ACTH production by the corticotroph cells *in vitro* was measured using a commercially available, nonisotopic, automatic, chemiluminescence immunoassay system (DPC Immulite, Los Angeles, CA). Intra- and interassay coefficients of variation were 5.6 and 7.8%, respectively.

Design of canine sst_2, sst_5, and D_2 primers

The sequences of the canine housekeeping gene hypoxanthine phosphoribosyltransferase (hprt) and sst_2, sst_5, and D_2 genes are available at the NCBI website (www.ncbi.nlm.nih.gov) with the following accession numbers: AY283372 (hprt), AY702068 (sst_2), XM_547202 (sst_5), and NM_001003110 (D_2). Primers and probes were designed with Primer Express software (Applied Biosystems, Foster City, CA) and ordered from Sigma Aldrich. Their sequences are depicted in Table 2.

qPCR

Expression analysis by qPCR was performed both on the 2×10^5 cells obtained via the isolation procedure as well as on a representative part of adenoma tissue that had been stored at −80 C directly postoperatively. For qPCR, we used a previously described method (34). In short, poly(A^+) mRNA was isolated from the corticotroph cells with the use of Dynabeads Oligo (Deoxythymidine)$_{25}$ (Dynal AS, Oslo, Norway). The poly(A^+) mRNA was eluted in H_2O (65 C) twice for 2 min each and used for cDNA synthesis in a Tris buffer [50 mM Tris-HCl (pH 8.3), 100 mM KCl, 4 mM dithiothreitol, and 10 mM $MgCl_2$] with 10 U ribonuclease inhibitor, 2 U avian myeloblastosis virus Super Reverse Transcriptase, and 1 mM of each deoxynucleotide triphosphate in a final volume of 40 μl. This was incubated for 1 h at 42 C, and the resulting cDNA was diluted 5-fold in 160 μl sterile H_20. One twentieth of the total cDNA library was used for quantification of hprt, sst_2, sst_5, and D_2 mRNA levels. The total reaction volume (25 μl) consisted of 10 μl cDNA and 15 μl TaqMan Universal PCR Mastermix (Applied Biosystems, Branchburg, NJ). Primers and probes were used at final concentrations of 300 nM (both primers) and 200 nM (probe). Real-time qPCR was performed in 96-well optical plates with the TaqMan Gold nuclease assay (Applied Biosystems, Roche) and the ABI Prism 7700 Sequence Detection System

TABLE 1. Clinical characteristics of canine patients included in this study

Case	Breed	Gender	Age (yr)	Body weight (kg)	Pit size[a] (mm)	P/B[b]	UCCR[c] ($\times 10^{-6}$)	DEX[d] (%)	ACTH[e] (pmol/liter)	α-MSH[f] (pg/ml)	Cortisol[g] (nmol/liter)	Remission[h]	Histopath diagnosis[i]	Immunohistochemistry
N1	Beagle	F	2	10.4	NA	NA	NA	NA	NA	NA	NA	NA	Normal	NA
N2	Beagle	F	2	9.2	NA	NA	NA	NA	NA	NA	NA	NA	Normal	NA
N3	Beagle	F	2	12.0	NA	NA	NA	NA	NA	NA	NA	NA	Normal	NA
N4	Beagle	F	2	8.4	NA	NA	NA	NA	NA	NA	NA	NA	Normal	NA
N5	Beagle	M	5	10.8	NA	NA	NA	NA	NA	NA	NA	NA	Normal	NA
C1	Sib. Husky	M	10.6	29.0	5–7-5	0.34	33.5	96.1	38.4	15.0	121	Yes	Adenoma	ACTH+, α-MSH+, GH−
C2	Dachshund	FC	8.4	7.7	5–5-4	0.35	26.5	86.4	17.5	6.5	61	Yes	Adenoma	ACTH+, α-MSH+, GH−
C3	Boxer	FC	5	32	9–9-8	0.42	241.0	96.8	12.2	16.5	265	Yes	Adenoma	ACTH+, α-MSH+, GH−
C4	Gold. Retriever	FC	10	28.4	10–11-11	0.56	31.0	−12.9	19.9	10.0	93	Yes	Adenoma	ACTH+, α-MSH+, GH−
C5	Vizla	MC	14	21.0	15–21-18	0.98	NA	NA	9.3	19.5	86.5	Yes	Adenoma	ACTH+, α-MSH+, GH−
C6	Stabyhoun	M	11	24.0	7–9-8	0.45	55.3	63.7	10.3	224	146.5	Yes	Adenohyp.	ACTH+, α-MSH+, GH+
C7	Bernese M. Dog	F	6.7	48.0	12–13-12	0.69	100.0	61.0	41.8	5	319	NA	Adenoma	ACTH+, α-MSH+/−, GH−
C8	Petit Bas. Gr.	MC	7.8	13.9	5–6-5	0.30	302.5	33.2	14.8	<5	414	Yes	Adenohyp.	ACTH+/−, α-MSH−, GH+
C9	Lab. Retriever	FC	8	36.5	12–12-12	0.56	30.7	88.3	22.2	20.5	192	Yes	Adenoma PI	ACTH+, α-MSH+/−, GH−
C10	Beagle	M	8	23.2	17–15-16	1.00	74.5	75.8	30.4	20	172	Yes	Adenoma	ACTH−, α-MSH−, GH−
C11	Mongrel	M	6.5	12.8	15–14-16	0.99	81	60.5	20.8	7	231.5	No[j]	Adenoma	ACTH+/−, α-MSH−, GH−
C12	Dachshund	M	11	6.7	8–10-10	0.55	207	76.1	21.0	<5	180.5	Yes	Adenoma	ACTH−, α-MSH−, GH−
C13	Beagle	MC	5.6	16.3	7–9-10	0.38	217.5	80.2	20.8	12.5	277.5	Yes	Adenoma	ACTH+, α-MSH+, GH−

Cases N1–N5 are normal dogs; C1–C13 are patients with CD. F, Female intact; FC, female castrated; M, male intact; MC, male castrated; NA, not available; PI, pars intermedia; Adenohyp., adenohypophysis; Sib. Husky, Siberian Husky; Gold. Retriever, Golden Retriever; Bernese M. Dog, Bernese Mountain Dog; Petit Bas.Gr., Petit Basset Griffon Vendéen; Lab. Retriever, Labrador Retriever.

[a] Pituitary size as measured on preoperative helical computed tomography (height-width-length).

[b] P/B $\times 10^{-2}$ mm^{-1} (P/B ≤0.31 indicates a normal-sized pituitary, P/B >0.31 indicates enlarged pituitary).

[c] Preoperative UCCR (reference $<10 \times 10^{-6}$); values are the mean of two morning urine samples with a 1-d interval.

[d] Preoperative degree of UCCR suppression after high-dose dexamethasone; 100 = complete suppression; 0 = no suppression of UCCR.

[e] Preoperative plasma ACTH (reference 1.1–18.7 pmol/liter); values are the mean of two samples with an interval of 10–15 min.

[f] Preoperative plasma α-MSH (reference <36 pg/ml); values are the mean of two samples with an interval of 10–15 min.

[g] Preoperative plasma cortisol (reference 11–136 nmol/liter); values are the mean of two samples with an interval of 10–15 min.

[h] Patient postoperative in remission at time of writing; *i.e.* UCCR $<5 \times 10^{-6}$ (yes/no).

[i] Diagnosis as stated by veterinary pathologist based on hematoxylin and eosin staining and IHC for ACTH, α-MSH, and GH.

[j] Recurrence at 4 months after hypophysectomy, after initial remission.

TABLE 2. Canine primer-probe sequences

Primer/probe	Sequence 5′–3′	Bases
sst_2		
Forward	GCCATACTATGACCTGACCAGCA	23
Reverse	TGTTGCCACACAATCCAATGA	21
Probe	FAM-TGCAGTCCTCACATTCATATATTT TGTGGTCTGC-TAMRA	34
sst_5		
Forward	TGCTGGTCATCTGCCTCTGTT	21
Reverse	GCCGGACGCCTTCACC	16
Probe	FAM-CCTGCTCATCGTGGTC-TAMRA	16
D_2		
Forward	TGGCCACGCTCGTCATG	17
Reverse	TGAATTTCCACTCACCTACCACC	23
Probe	FAM-CCTGGGTTGTCTACCTG-TAMRA	17
hprt		
Forward	GCTTGCTGGTGAAAAGGACC	20
Reverse	GAATTTCAAATCCAACAAGTCAGGT	25
Probe	FAM-CTCGAAGTGTTGGCTATA-TAMRA	18

(PerkinElmer, Foster City, CA). After two initial heating steps at 50 C (2 min) and 95 C (10 min), samples were subjected to 40 cycles of denaturation at 95 C (15 sec) and annealing at 60 C (60 sec). All samples were assayed in duplicate. Values were normalized against the expression of the housekeeping gene hprt. Dilution curves were constructed to calculate PCR efficiencies (E) for every primer-probe set (35). Efficiencies were as follows: sst_2 2.01, sst_5 1.77, D_2 1.96, and hprt 1.84. Estimated copy numbers were calculated using the comparative threshold method with efficiency correction, as described previously (36). To exclude genomic DNA contamination in the RNA, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. To exclude contamination of the PCR mixtures, the reactions were also performed in the absence of cDNA template, in parallel with cDNA samples.

Assessing purity of corticotroph cell population

Three steps were taken to secure the purity of the examined corticotroph adenoma tissue. First, the veterinary surgeon provided us only with pituitary tissue that was macroscopically adenomatous. When the surgeon assessed the pituitary tissue to be a mix of adenomatous and unaffected tissue, this was specifically noted. Second, a part of the isolated cells (1.0×10^5) was used to check for ACTH immunopositivity on freshly prepared cytospins (see below for methods). Only isolated cell populations with significant ACTH immunopositivity were eligible for analysis. As a third and final step, the expression of GH and proopiomelanocortin (POMC) mRNA was analyzed in all samples with Bio-Rad My-IQ detection system (IQ SYBR Green Supermix and My-IQ; Bio-Rad, Veenendaal, The Netherlands) with final primer concentrations of 400 nM according to previously published protocols (37). For GH and POMC, the ribosomal protein S19 (rps-19) was used as a reference gene (38). Ratios of GH/POMC mRNA expression were established in normal anterior pituitary (NAP) cells (N1–5) and compared with those in the corticotroph adenoma samples (C1–13).

Neurogenic differentiation factor D1 (NeuroD1) expression

To investigate the possible origin of the corticotroph adenoma (anterior *vs.* intermediate lobe), we also assessed NeuroD1 mRNA expression in all samples, using the same qPCR protocol as for GH and POMC and with rps-19 as reference gene. NeuroD1 is a transcription factor that promotes POMC expression and is a corticotroph marker in mice, dogs, and humans (39, 40). It is highly expressed in the normal canine anterior lobe but not in the intermediate lobe (41).

Dexamethasone and sst_2 mRNA expression

To study the effects of glucocorticoids on sst_2 expression, isolated corticotroph cells were plated at a density of 100,000 cells per well and cultured for 72 h in the presence or absence of the glucocorticoid dexamethasone (10 nM), the glucocorticoid receptor antagonist RU-486 (100 nM), or their combination. After 72 h, cells were lysed and mRNA expression levels of sst_2 and hprt were determined. All experimental conditions were performed in quadruplicate.

IHC: paraffin-embedded tissue and cytospins

The expression of ACTH and sst_2 was assessed in representative adenoma tissue by means of IHC according to a previously published method (42). Formalin-fixed, paraffin-embedded corticotroph adenoma tissues were cut (5 μm), deparaffinized, rehydrated, heated in citrate buffer (pH 6.0) for 20 min at 100 C for antigen retrieval, and incubated with the following primary antibodies: anti-ACTH (Santa Cruz Biotechnology, Santa Cruz, CA; mouse monoclonal, 1:100, 1 h at room temperature) and anti-sst_2 (Gramsch Laboratories, Schwabhausen, Germany; rabbit polyclonal, 1:2000, overnight 4 C). This was followed by a 30-min incubation at room temperature with poly-AP-goat antimouse/rabbit IgG from PowerVision+ (ImmunoVision Technologies Co., Brisbane, CA) and a 30-min incubation in New Fuchsin solution. Slides were counterstained with hematoxylin and eosin and coverslipped. Negative controls included omission of the primary antibody and preabsorption with an immunizing receptor peptide (100 nM) for the sst_2 polyclonal antibody. Three different commercially available antibodies against the human D_2 and two against the human sst_5 receptor were tested on canine NAP tissues and on a number of canine corticotroph adenomas. Unfortunately, none of these antibodies resulted in specific immunohistochemical staining.

To check for corticotroph purity of the adenoma specimen obtained at surgery (see above), cytospins of freshly isolated adenoma cells were made using a Cytospin 4 machine (Thermo Shendon Ltd., Astmoor, UK), in which 2×10^4 cells were spun onto adhesive microscopic slides (Starfrost, Braunschweig, Germany). Subsequently, they were air dried and fixed in acetone for 10 min, and next, a similar IHC protocol as described above was used with an anti-ACTH antibody dilution of 1:600. In these cytospins, we counted the percentage of ACTH-positive cells as a measure of the percentage of corticotrophs in our isolated cell population.

Test substances

Test substances were obtained from Novartis Pharma AG, Basel, Switzerland [octreotide (OCT) and PAS]; Sigma Aldrich (RU-486); Pharmacia, Milan, Italy (CAB); and the Erasmus Medical Center pharmacy (dexamethasone and CRH).

Statistical analyses

All data were analyzed with GraphPad Prism software (San Diego, CA). Data on hormone release are expressed as mean ± SEM. All experiments were run in quadruplicate. Overall differences between treatment groups were determined by ANOVA. In case of significant differences found by ANOVA, a multiple comparison between groups was performed with a Newman-Keuls test. Correlation analyses were performed between the expression levels of NeuroD1, sst, or D_2 receptor subtypes and/or corresponding preoperative hormone levels by determining Spearman's correlation coefficients. P values < 0.05 were considered statistically significant.

Results

Study population follow-up

Remission of hypercortisolism occurred in 12 of the 13 dogs and was confirmed by resolution of clinical signs and UCCR values less than 5×10^{-6} within 8 wk after hypophysectomy. In one dog (C11), hypercortisolism recurred 4 months postoperatively. One other dog was lost to follow-up (C7). Histopathology revealed pituitary adenoma in 11 of 13 cases, with an adenoma originating from the pars intermedia in one case (C9). Immunostaining was positive for ACTH in 11 of 13 cases (Table 1).

Purity of obtained corticotroph tissue

Macroscopically pure adenoma tissue was identified by the surgeon in nine of 13 cases. In the remaining cases, the resected tissue was a mixture of adenoma and unaffected (preexistent) pituitary tissue. Cytospins that were prepared from the isolated corticotroph cells showed variable but significant ACTH immunoreactivity in all cases that were analyzed (Table 3).

GH and POMC mRNA expression was determined in the five NAP and in the 13 adenomas (Table 3). The mean (± SEM) POMC/GH ratio in the five NAP cases was 0.36 ± 0.18. We defined pure corticotroph adenomas as having a POMC/GH mRNA ratio of at least 10 times higher than the POMC/GH mRNA ratio observed in NAP. In this way, eight of 13 adenomas were classified as pure adenomas and five of 13 adenomas as a mixture of adenoma and unaffected (*i.e.* nonpure) pituitary tissue. Four of the latter five adenomas had been classified macroscopically by the surgeon as being a mixture. One case (C13) was assessed by the surgeon as pure adenoma, but the POMC/GH mRNA ratio *in vitro* was low, indicating nonpure pituitary tissue.

mRNA expression: sst, D_2, and NeuroD1

In the corticotroph adenoma cells, which were obtained after cell dispersion *in vitro*, there was a strong but highly variable expression of the sst_2 receptor subtype (median, 1.90; range, 0.22–26.28) with two adenomas (C1 and C6) showing very high sst_2 expression levels (Fig. 1). D_2 was moderately expressed (median, 0.75; range, 0.00–8.07), and sst_5 was expressed at very low levels (median, 0.02; range, 0.00–0.49). These results were confirmed in similar but independent experiments with RNA that was extracted from the primary adenoma tissue that had been stored directly postoperatively at −80 C. In these experiments, a similar mRNA expression pattern was observed (data not shown). For comparison, expression levels in the NAP were as follows (median; range): sst_2 (7.98; 3.81–18.7), sst_5 (0.30; 0.08–0.66), and D_2 (0.96; 0.45–2.98). The anterior pituitary marker NeuroD1 was variably expressed among the adenomas with a median value of 0.43×10^{-2} (range, 0.04–9.67×10^{-2}), which was higher than that of NAP (median, 0.21×10^{-2}; range, $0.08–0.23 \times 10^{-2}$; Table 3). No significant correlations were found between NeuroD1 and sst-D_2 receptor subtype expression or with preoperative hormone levels (Spearman's correlation coefficients: $P > 0.05$).

In vitro culture data

For seven pure corticotroph adenomas, we were able to measure the effects of DA/SS analogs on ACTH inhibition *in vitro*. Mean basal ACTH production in these adenomas was 86 pmol/liter at 4 h (range, 33–188), 222 pmol/liter at 24 h (range, 56–471), and 591 pmol/liter at 72 h (range, 88–1240). Stimulation with 10 nM CRH induced a mean 2.0-fold increase (range, 0.7–3.9) in ACTH production at 4 h compared with basal. In all adenomas combined, the sst_2-preferring agent OCT was most effective at inhibiting 4-h CRH-induced ACTH release (−27%, $P < 0.01$ *vs.* control), whereas the multiligand SS analog PAS (SOM230) (−18%, $P < 0.05$) and the D_2-agonist CAB (−13%, $P < 0.05$) were less effective (Fig. 2A). All compounds were used at the 10 nM concentration. Combining CAB with either OCT or PAS did not increase ACTH inhibition compared with OCT or PAS alone (OCT+CAB, −23%, $P < 0.05$ *vs.* control; PAS+CAB, −20%, $P < 0.05$). Of note, the two adenomas with the highest sst_2 mRNA expression (C1 and C6) were also most responsive to OCT (10 nM) treatment in terms of 4-h CRH-induced ACTH inhibition: C1, OCT −67%, $P < 0.001$ (Fig. 2B); C6, OCT −74%, $P < 0.001$ (Fig. 2C). The other five adenomas (C4, C5,

TABLE 3. mRNA expression data and IHC cytospins

Case	Tissue[a]	ACTH+[b]	POMC/GH[c]	Classification[d]	NeuroD1[e]
N1	N	NA	0.35	Normal	0.22
N2	N	NA	0.10	Normal	0.16
N3	N	NA	0.07	Normal	0.08
N4	N	NA	0.22	Normal	0.21
N5	N	NA	1.05	Normal	0.23
C1	C	NA	231,495.07	Pure adenoma	0.04
C2	C/N	3+	3.58	Nonpure	0.43
C3	C/N	1+	0.02	Nonpure	0.10
C4	C	4+	5,920.75	Pure adenoma	2.27
C5	C	3+	149.43	Pure adenoma	0.93
C6	C	3+	11.45	Pure adenoma	0.29
C7	C/N	3+	0.17	Nonpure	0.63
C8	C/N	NA	0.03	Nonpure	0.09
C9	C	4+	548.86	Pure adenoma	0.06
C10	C	NA	12.82	Pure adenoma	0.17
C11	C	3+	856.95	Pure adenoma	9.67
C12	C	1+	6,230.95	Pure adenoma	4.43
C13	C	3+	1.82	Nonpure	0.50

Cases N1–N5 are normal dogs; C1–C13 are patients with CD. NA, Not available.

[a] Macroscopic appearance of resected tissue as judged by veterinary surgeon. C, Pure adenoma tissue; C/N, mixture of adenoma and unaffected tissue; N, unaffected tissue.

[b] Percentage ACTH-positive cells on cytospin: 1+ (0–10%), 2+ (10–20%), 3+ (20–30%), and 4+ (>30%).

[c] POMC/GH mRNA ratio in normal anterior pituitary cells (N1–N5) and in corticotroph adenoma cells (C1–C13).

[d] Classification of tissue: normal (*i.e.* unaffected anterior pituitary tissue), pure adenoma tissue, or nonpure (mixed adenoma-unaffected) tissue.

[e] NeuroD1/rps-19 mRNA ($\times 10^{-2}$) expression.

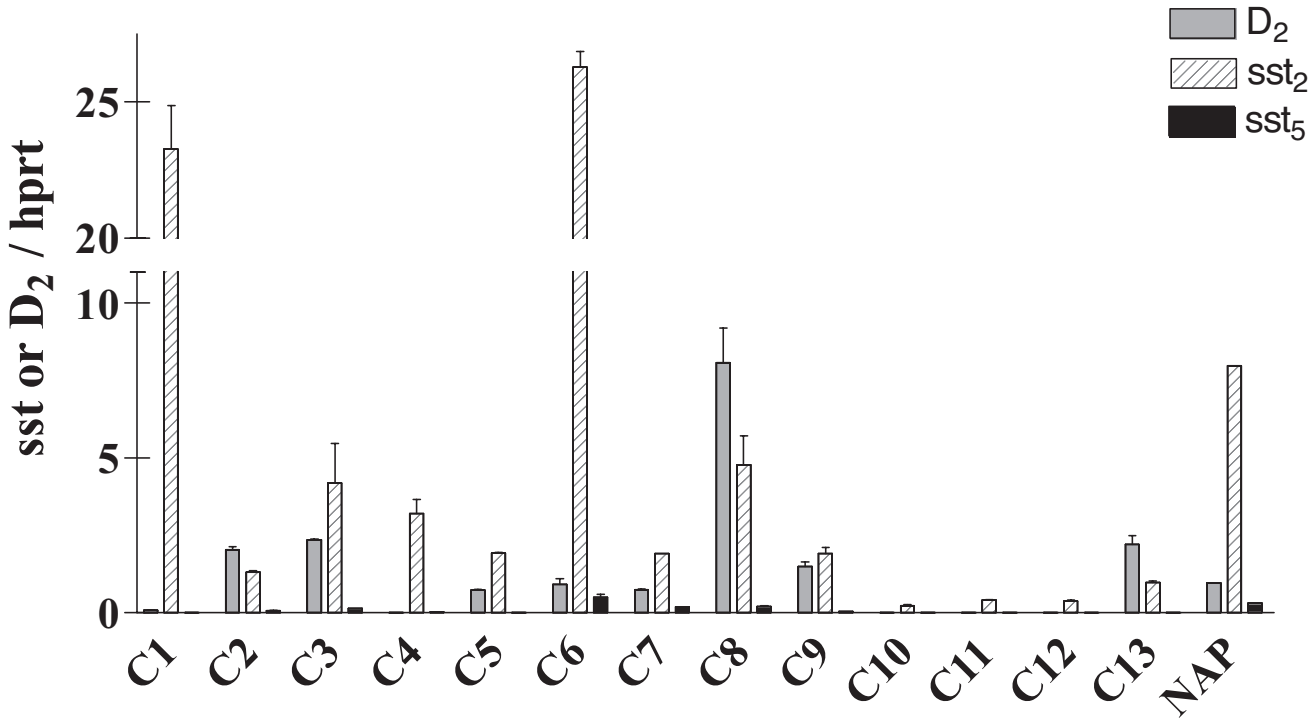

FIG. 1. Overview of sst_2, sst_5, and D_2 mRNA expression in canine corticotroph pituitary adenomas (C1–C13) and in NAP. All expression levels are normalized against the housekeeping gene hprt. Values for C1–C13 represent the mean of two duplicate measurements ± SEM. For comparison, the median expression level in NAP (n = 5) is depicted.

C9, C11, and C12) showed minor to moderate (10–30%) ACTH inhibition in response to the different compounds.

Parallel to this, we investigated ACTH inhibition in these adenomas without CRH stimulation. At the 24-h time point, a similar pattern of response to DA and SS analogs was observed. Data for all adenomas combined were as follows: OCT −20% ($P < 0.001$ *vs.* control), PAS −13% ($P < 0.05$), and CAB −9% ($P > 0.05$). In these experiments without CRH stimulation, adding CAB to OCT or PAS increased the overall ACTH inhibition: OCT+CAB, −24% ($P < 0.001$ *vs.* control), and PAS+CAB, −20% ($P < 0.001$) (Fig. 2D). Similar patterns of inhibition were observed after 72 h, although average levels of ACTH inhibition were lower at this time point (data not shown).

IHC

In normal canine pituitary tissue, sst_2 was expressed in the anterior pituitary, but immunoreactivity for sst_2 was especially strong in cells of the intermediate lobe (Fig. 3). The staining pattern was primarily cytoplasmic and absent with omission of the primary antibody or when coincubated with an immunizing peptide. In a subset of patients (n = 5), we were able to perform IHC for sst_2 on the corticotroph adenoma tissue that was formalin fixed and paraffin embedded directly after surgery. For these adenomas, the results of IHC for sst_2 expression corresponded well with the previously described mRNA data. In one of the tumors with a very high sst_2 mRNA expression (C1), a strong overall sst_2 staining was observed with clear colocalization of sst_2 and ACTH immunoreactivity (Fig. 4), whereas the other corticotroph adenomas showed staining of minor intensity (C4 and C5) or only of isolated cells (C2 and C3). Due to unavailability of canine-specific antibodies, we were not able to test for sst_5 or D_2 immunopositivity in these tissues.

Dexamethasone and sst_2 mRNA expression

To explore potential regulation of receptor subtype expression by glucocorticoids, we investigated the effects of the synthetic glucocorticoid dexamethasone (DEX) on sst_2 mRNA expression in two primary corticotroph cultures (C4 and C12) with a sufficiently high cell yield that allowed us to perform these additional experiments. Treatment with 10 nM DEX for 72 h caused increased sst_2 mRNA expression in both adenomas with an average increase of 61% ($P < 0.05$ *vs.* control; Fig. 5), with C4 +51% ($P > 0.05$) and C12 +71% ($P < 0.05$). Addition of the glucocorticoid antagonist RU-486 (100 nM) abolished these effects. The effects of DEX could not be investigated for sst_5 and D_2, because the expression levels of these subtypes were too low in these particular adenomas.

Discussion

Canine corticotroph adenomas resected during transsphenoidal surgery constitute a new and interesting source for retrieving considerable amounts of valuable primary corticotroph tissue. This primary tissue can be of great value for research regarding pituitary developmental processes as well as etiology, diagnosis, and therapy of pituitary disorders (43). Due to the high incidence of CD in dogs, surgical specimens of fresh adenoma tissue become available on a routine basis and have a high average yield in terms of viable corticotroph adenoma cells. Furthermore, these cells remain

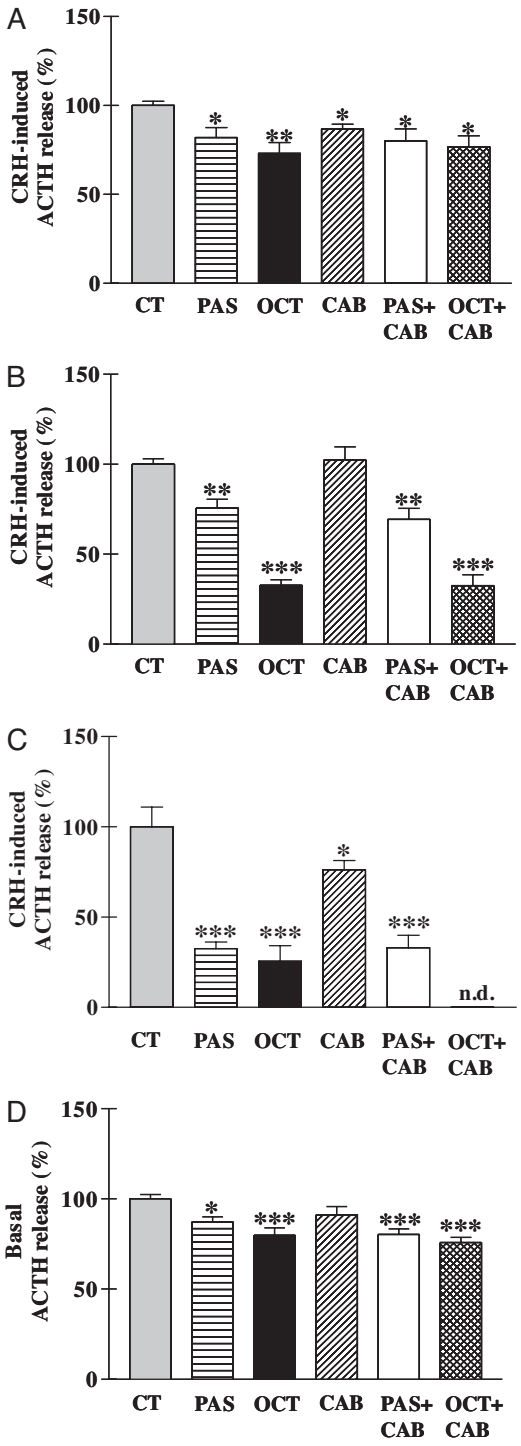

FIG. 2. ACTH inhibition in canine corticotroph adenomas. A–C, Primary corticotroph cells were cultured and stimulated for 4 h with 10 nM CRH in the presence or absence of 10 nM PAS, 10 nM OCT, 10 nM CAB, or their combination. Data are shown for all adenomas combined (A), adenoma C1 (B), and adenoma C6 (C). D, Basal ACTH production after 24 h in the presence or absence of the same compounds as above (data for all adenomas combined). After 4 or 24 h, respectively, media were collected and ACTH levels determined. All experimental conditions were performed in quadruplicate. Values represent percent change ± SEM relative to control (CT). Control was CRH alone (for CRH data) or untreated cells (for basal data). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control; n.d., not determined.

viable in culture, produce ACTH in significant amounts, are CRH responsive to a variable degree, and can respond to commonly used agonists *in vitro*. The fulfillment of all of these criteria makes canine corticotroph adenomas a feasible and readily used model for the study of (human) CD.

The main objective of our present study was to evaluate the expression and functional significance of DA (D_2) and SS receptor subtypes (sst_2 and sst_5) within these canine corticotroph adenomas. These receptor subtypes are the main focus of much of the current research into human CD, and agonists that target these receptor subtypes have already been used in clinical studies with promising results (6, 8). From this perspective, canine corticotroph adenoma tissue could constitute a useful tool to further explore efficacy and mechanism of action of novel SS or DA compounds for future use in human CD.

Despite the many striking similarities in etiology and clinical presentation between human and canine CD, canine corticotroph adenomas differ clearly from their human counterparts in terms of SS and DA receptor expression patterns. Canine corticotroph adenomas mainly express sst_2, whereas D_2 and especially sst_5 are expressed at much lower levels. The predominance of sst_2 is observed at the mRNA level, as demonstrated by qPCR, and confirmed at the protein level by immunohistochemical studies. In agreement with this, the sst_2-preferring agonist OCT is the most efficacious agent in inhibiting ACTH release in both basal and CRH-stimulated conditions, whereas the multiligand SS analog PAS is significantly less effective. The lower efficacy of PAS compared with OCT is readily explained by its 2.5-fold lower binding affinity for the sst_2 receptor (IC_{50} 1.0 *vs.* 0.38 nM, respectively) (44) in combination with the low overall expression of sst_5 in canine corticotroph adenomas. The D_2 agonist CAB shows some efficacy in the seven cultured adenomas combined, albeit lower than OCT and PAS. This finding is in line with the lower D_2 mRNA expression compared with sst_2 observed in this study.

Nonetheless, this modest level of D_2 receptor expression could still prove to be of functional value. In a recent study by Castillo *et al.* (45), dogs with CD were treated with CAB (0.07 mg/kg/wk) for 1 yr, which resulted in an overall response rate of 42.5%. One factor that could explain this observed difference between the *in vitro* and clinical efficacy of CAB could be the duration of treatment. It is known from studies in human patients with CD that it can take up to 3 months before the maximal cortisol-inhibiting effects of CAB are observed (6). In this respect, our *in vitro* data on ACTH inhibition after 4–72 h may not necessarily reflect the full potential of CAB as a drug in canine CD. On the other hand, the high levels of sst_2 expression both on the mRNA and the protein level, in combination with the superior efficacy of OCT in cultured canine corticotroph adenomas, suggest an even stronger role of this receptor subtype as a therapeutic target. Based on our findings, a clinical study to investigate the effects of an sst_2-preferring compound such as OCT on ACTH and cortisol levels in canine CD could be of great interest to see whether superior response rates could be achieved with the use of such compounds compared with those obtained with CAB. In addition to this, it would be very interesting to study whether combined targeting of sst_2 and

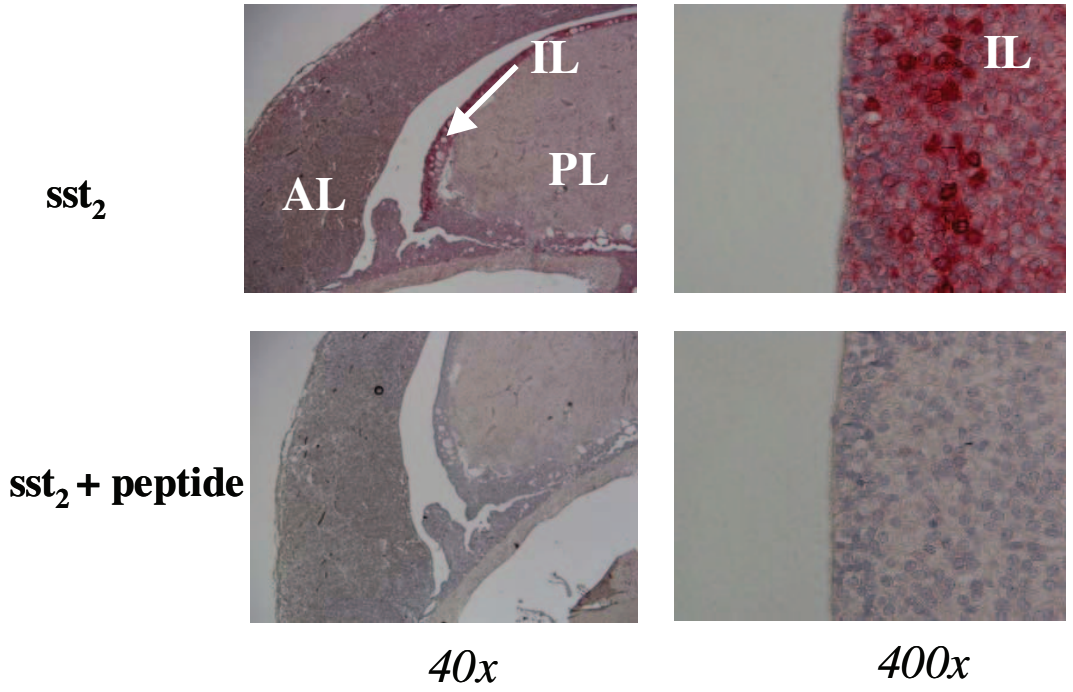

FIG. 3. IHC for sst_2 expression in the normal canine anterior pituitary. *Top left* (magnification, ×40), strong sst_2 expression in the anterior lobe (AL) and the intermediate lobe (IL, *arrow*) but not in the posterior lobe (PL); *top right* (magnification, ×400), cytoplasmic staining for sst_2 in individual cells of the intermediate lobe; *bottom left* (×40) and *right* (×400), no staining in negative control with immunizing receptor peptide.

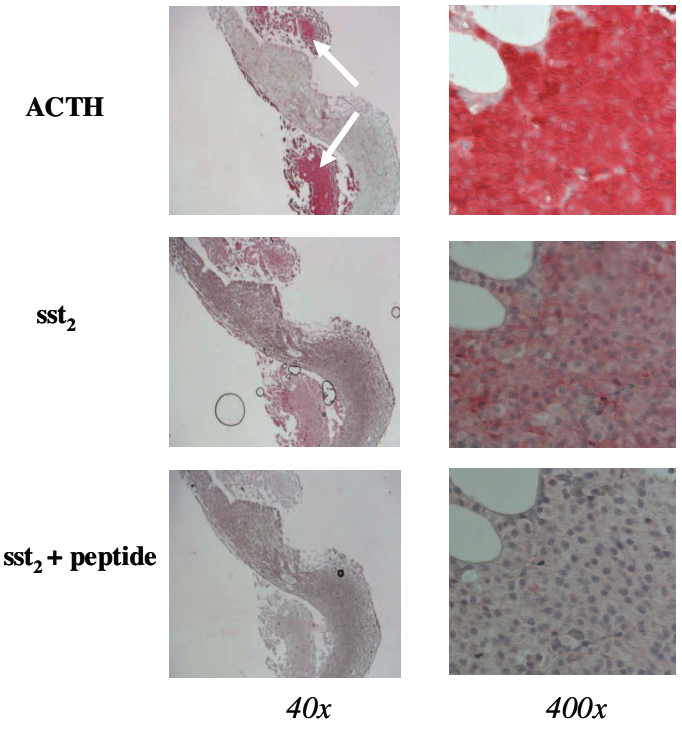

FIG. 4. IHC for sst_2 expression in canine corticotroph adenoma C6. *Top*, Strong ACTH expression in the adenomatous tissue (*arrow*) [magnification, ×40 (*left*) and ×400 (*right*); *middle*, sst_2 expression is evident in the areas of ACTH-positive adenoma tissue (×40 and ×400); *bottom*, no staining in negative control with immunizing receptor peptide (×40 and ×400).

D_2 receptors, either by cotreatment with the individual SS/DA analogs or by the use of novel chimeric SS-DA molecules could result in even higher clinical efficacy.

To return to our original research question, the receptor expression pattern observed in canine adenomas is remarkably different from the one observed in human corticotroph adenomas, where sst_5 and D_2 are the predominant receptor subtypes and sst_2 expression is generally low. The reasons for

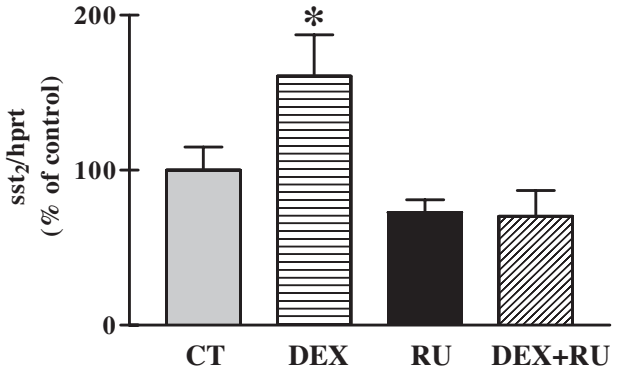

FIG. 5. Glucocorticoid regulation of sst_2 mRNA expression. Adenoma cells of C4 and C12 were cultured in the absence or presence of the glucocorticoid dexamethasone (DEX) (10 nM) and/or the glucocorticoid receptor antagonist RU-486 (RU) (100 nM). After 72 h, cells were lysed and mRNA expression levels of sst_2 and housekeeping gene hprt were determined by qPCR. The hprt expression levels did not vary among treatment groups. All experimental conditions were performed in quadruplicate. Values represent percent change ± SEM relative to control. *, $P < 0.05$ *vs.* control (CT).

this dissimilarity between canine and human corticotroph adenomas are yet unknown. One important factor, however, appears to be the difference in regulation by glucocorticoids of receptor subtype expression. Down-regulation of sst_2 expression by glucocorticoids has been demonstrated in murine corticotroph AtT20 tumor cells and is also thought to explain the low sst_2 expression in human corticotroph adenomas (5, 46, 47). Striking, therefore, was the observation in our study that this glucocorticoid-induced down-regulation did not occur in canine corticotroph adenomas. In fact, treatment of the canine corticotroph cells *in vitro* with dexamethasone increased the expression of the sst_2 receptor, as was observed in two different adenomas. From a future perspective, it would be interesting to see whether these differences can be ascribed to the 7% inhomology between the canine and the human sst_2 genetic sequence, because it is possible that this genomic variation is also present in areas within the human sst_2 gene that are known to contain glucocorticoid-responsive elements.

It is important to emphasize that sst and D_2 are not the only receptors that have been linked to regulation of ACTH secretion in corticotroph cells. Receptors such as the retinoic acid receptor (RAR) and peroxisome-proliferator-activated receptor-γ (PPARγ) have also been shown to decrease ACTH regulation in different *in vitro* and rodent models and have therefore been implicated as potential new targets for medical therapy of CD in humans (48, 49). Most notably, retinoic acid was used in a recent clinical study in dogs with CD and showed significant clinical efficacy (50). In this respect, it would be very interesting to evaluate canine corticotroph adenomas for the presence and distribution of novel drug targets such as retinoic acid receptor and peroxisome-proliferator-activated receptor-γ and to see whether correlation is higher between canine and human CD for these receptors than for SS and DA. These investigations could help to fully evaluate the potential of canine CD as a direct animal model for human CD.

In conclusion, canine corticotroph adenomas obtained after transsphenoidal surgery, provide a model to study corticotroph cell (patho)physiology due to the high yield of viable, primary tissue that retains most of its corticotroph features *in vitro*. Some distinct differences do exist, however, between human and canine corticotroph adenomas in terms of sst and D_2 receptor expression patterns and their responses to SS and DA agonists *in vitro*. These differences should be taken into account when using dogs with CD as a model to evaluate efficacy of novel SS analogs and DA agonists for future use in human CD.

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