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Expression of regulatory neuropeptides in the hypothalamus of red deer (*Cervus elaphus*) reveals anomalous relationships in the seasonal control of appetite and reproduction

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ABSTRACT

Red deer are seasonal with respect to reproduction and food intake, so we tested the hypothesis that their brains would show seasonal changes in numbers of cells containing hypothalamic neuropeptides that regulate these functions. We examined the brains of male and female deer in non-breeding and breeding seasons to quantify the production of kisspeptin, gonadotropin inhibitory hormone (GnIH), neuropeptide Y (NPY) and γ-melanocyte stimulating hormone (γ-MSH – an index of pro-opiomelanocortin production), using immunohistochemistry. These neuropeptides are likely to be involved in the regulation of reproductive function and appetite. During the annual breeding season there were more cells producing kisspeptin in the arcuate nucleus of the hypothalamus than during the non-breeding season in males and females whereas there was no seasonal difference in the expression of GnIH. There were more cells producing the appetite stimulating peptide, NPY, in the arcuate/median eminence regions of the hypothalamus of females during the non-breeding season whereas the levels of an appetite suppressing peptide, γ-MSH, were highest in the breeding season. Male deer brains exhibited the converse, with NPY cell numbers highest in the breeding season and γ-MSH levels highest in the non-breeding season. These results support a role for kisspeptin as an important stimulatory regulator of seasonal breeding in deer, as in other species, but suggest a lack of involvement of GnIH in the seasonality of reproduction in deer. In the case of appetite regulation, the pattern exhibited by females for NPY and γ-MSH was as expected for the breeding and non-breeding seasons, based on previous studies of these peptides in sheep and the seasonal cycle of appetite reported for various species of deer. An inverse result in male deer most probably reflects the response of appetite regulating cells to negative energy balance during the mating season. Differences between the sexes in the seasonal changes in appetite regulating peptide cells of the hypothalamus present an interesting model for future studies.

1. Introduction

Red deer exhibit pronounced seasonal cycles of reproductive activity and live weight (Loudon et al., 1989; Webster et al., 1996) which makes them an excellent model species for investigation of neuroendocrine factors involved in the regulation of seasonal changes in reproduction and appetite. Kisspeptin is a potent stimulator of gonadotropin releasing hormone (GnRH) secretion and plays a major role in the central control of reproduction in mammals (Clarke et al., 2011; Okamura et al., 2013). Evidence is also gathering to indicate a role for another RF-amide, gonadotropin inhibitory hormone (GnIH), which acts inversely as a switch between reproduction and appetite (Clarke et al., 2012). Food intake is also stimulated by neuropeptide Y (NPY) (Sartin et al., 2010; Mercer et al., 2011) and inhibited by melanocortins that are products of pro-opiomelanocortin (POMC) precursor in neurons within the arcuate nucleus of the hypothalamus in ruminants (Sartin et al., 2010; Marston et al., 2011; Clarke, 2014).

The aim of this study was to compare the levels of kisspeptin, GnIH, NPY and γ-MSH, a pro-opiomelanocortin (POMC)-derived peptide, in the relevant hypothalamic regions of the brains of male and female red deer during spring and autumn, as revealed by immunohistochemistry. These two contrasting seasons are times when both food intake and reproductive activity differ in deer, so the relative activity of these peptides in relation to these functions is important. These findings have been based primarily on studies of sheep, so it is likely that evidence from a highly seasonal deer

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species would strengthen the case for their applicability to ruminants in general. We hypothesise that the hypothalamic regions of deer will display seasonal differences in the numbers of cells containing the feed intake regulating and reproductive peptides, and these will be the inversely related.

2. Materials and methods

2.1. Animals

Sixteen mature red deer (*Cervus elaphus* subspecies *scoticus*) – stags and hinds – were taken from the Lincoln University Research Farm, Canterbury, New Zealand. They were grazed on pasture consisting of predominantly rye-grass and white clover, with water available *ad libitum* and live weight and body condition score (on a scale of 0–5, Audigé et al., 1998) was recorded at weekly intervals for 3–8 weeks. Jugular blood samples were also collected at these times. The blood was centrifuged to obtain plasma, which was frozen at −20 °C until assayed for LH. Half of the animals from each gender group (i.e. 4 males and 4 females) were selected at random to be killed humanely either in autumn (April/May); during the breeding season (rut), or in summer (November/December) during the non-breeding season. The animals were euthanized by an overdose of pentobarbital sodium after which the heads were removed then perfused through the external carotid arteries with 3 l of 0.9% w/v saline solution followed by 1 l of 10% formalin in normal saline. The brains were stored in the formalin solution until processing for immunohistochemistry as described below. All animal procedures were approved by the Lincoln University Animal Ethics Committee.

2.2. Plasma LH assay

An assay that was originally developed for ovine LH (Lee et al., 1976) was used to measure this hormone in plasma samples from the stags. All samples were included in a single assay and we verified that measurements in deer plasma diluted in parallel with the ovine standard.

2.3. Immunohistochemistry

The hypothalami were dissected from the deer brains and then placed in 30% sucrose in phosphate buffered saline solution (PBS) until they sank. The tissue blocks were then rinsed in PBS, frozen on powdered dry ice, wrapped in plastic paraffin film (Parafilm® M, Sigma-Aldrich, St Louis, MO, USA) and stored at −20 °C until coronal sections (30 μm) were cut on a cryostat. Sections were collected into tissue culture wells containing cryoprotectant and stored at −20 °C prior to use.

The sections were subsequently processed for 3,3′-diaminobenzidine (DAB) immunohistochemistry. Anatomically matching sections representing the arcuate nucleus were selected for kisspeptin, NPY and γ-MSH immunostaining and sections from the dorsomedial nucleus (DMN) were taken for GnIH immunostaining, from each animal (4 per group). For each peptide in either nucleus, sections from the rostral, middle, and caudal regions were selected. The sections were mounted onto Superfrost™ slides (Thermo Fisher Scientific Inc., Waltham, MA, USA) and dried overnight.

Kisspeptin cells were detected with a polyclonal rabbit antibody raised against mouse kisspeptin-10 (a gift from Dr. A. Caraty, INRA, Nouzïlly, France) at a dilution of 1:2000. This antibody and the others named below were applied respectively to the sections in 0.1 M Tris-buffered saline solution (TBS) containing 5% normal goat serum, 0.3% Triton X-100 and 0.1% NaN_3, as described previously (Goodman et al., 2007; Smith et al., 2008b). To detect NPY, a polyclonal rabbit antibody was used (gift from Dr. A. Lawrence, University of Melbourne, Australia) at a dilution of 1:2000, with specificity also determined previously in ovine tissues (Barker-Gibb and Clarke, 1996). POMC-producing cells were identified using a guinea pig γ-MSH antibody at a dilution of 1:4000 (Antibodies Australia, Melbourne, Australia), with specificity as previously described in ovine tissues (Goodman et al., 2007). Cells containing GnIH (RFRP) were detected using a rabbit antibody PAC 123a (a gift from Dr. G. E. Bentley, University of California, Berkley, CA, USA) at a dilution of 1:1000, with specificity previously determined in ovine tissues (Smith et al., 2008a).

For detection of kisspeptin, NPY, POMC and GnIH-containing neurons, antigen retrieval was performed using 0.1 M citrate buffer (pH 6) in a microwave oven at 1000 W (twice for 5 min each time), cooling for 30 min and subsequent washes in TBS. This was followed by elimination of endogenous peroxidase with 3% hydrogen peroxide for 20 min. After washes in TBS, blocking solution containing 5% normal goat serum and 0.3% Triton X-100 in TBS, was applied to the sections for 1 h at room temperature. The sections were then incubated for 72 h at 4 °C with the relevant primary antibody. Subsequently, the sections were washed in TBS, and incubated with biotinylated secondary antibody for 2 h at room temperature; goat-anti-rabbit (DAKO, Carpinteria, CA, USA) for kisspeptin, NPY and GnIH detection at a dilution of 1:200 and goat anti-guinea pig (Vector Laboratories Inc., Burlingame, CA, USA) for γ-MSH detection at a dilution of 1:400. Following washes in TBS, sections were incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA, USA) for 1 h at room temperature. Visualisation was with diaminobenzidine (DAB) (Sigma Aldrich, St Louis, MO, USA), to produce a brown reaction product in the cytoplasm of immuno-labelled cells.

The number of immuno-labelled cells was recorded for the rostral, mid and caudal regions of the arcuate nucleus in the case of kisspeptin, NPY and γ-MSH immunostaining, and also the rostral, mid and caudal regions of the DMN in the case of GnIH immunostaining, and was summed for each animal. The number of cells per region and in total for deer in both groups (breeding and non-breeding season) were averaged to produce means (±SEM). Because we used antigen-retrieval, no measurement was made of the intensity of immunostaining.

2.4. Data analysis and statistics

Prior to statistical analyses, homogeneity was assessed using the Levene's test and the total number of NPY and ɣ-MSH cells were subjected to log transformation. Total cell numbers were analysed using a univariate ANOVA incorporating both sex and season. A repeated measure ANOVA was used to analyse seasonal effects on the number of cells expressed across the rostral, middle and caudal extent of each nucleus. In each case, post hoc analyses were performed using a one way ANOVA incorporating the least significant difference as test statistic. LH data were analysed as a time-series for repeated measures, also using least significant difference at the test statistic.

3. Results

3.1. Live weight, body condition score, LH, ovaries, pelage and antler stage

Autumn-killed animals (breeding season), especially stags, were losing weight at the rate of 550 g/day for 35 days in stags and 51 g/day for 15 days in hinds. In contrast, Spring-killed animals (non-breeding season) were gaining live weight; 740 g/day for