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Substance P-Immunoreactive Cells in the Ovine Pars Tuberalis

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Abstract

The pars tuberalis (PT) is a distinct subdivision of the anterior pituitary gland that plays a central role in regulating seasonal prolactin release. In sheep, there is compelling evidence that seasonal changes in light, transformed into a melatonin signal, are interpreted by the PT to modulate the release of a factor which affects prolactin release. The identity of this factor(s) is unknown but has been preemptively called ‘tuberalin’. In the present study, we report on an initial immunocytochemical investigation where we have identified that many ovine PT cells are immunoreactive for the tachykinin substance P (SP). Few cells in the pars distalis immunoreact for SP. The SP-immunoreactive cells did not colocalize with β-luteinizing hormone. RT-PCR confirmed the presence of preprotachykinin A mRNA in the PT. We hypothesize that SP, and possibly other preprotachykinin A-derived tachykinins, may play a role in the seasonal regulation of prolactin secretion in sheep.

Keywords

Substance P; Neurokinin; Tachykinin; Prolactin; Seasonal rhythms

Introduction

Most animals exhibit circadian and seasonal rhythms. Circadian rhythms are regulated by the suprachiasmatic nucleus [1], but how and where seasonal rhythms are controlled remains unsolved. Almost all seasonal rhythms are driven by photoperiod. Photoperiodic information is translated by the pineal gland into a biochemical signal, melatonin, secreted during the night [2]. Thus, the duration of melatonin release is an accurate representation of the length of the night and, thereby, season [3]. Williams and Morgan [4] revealed melatonin binding sites in several areas of the rat brain but, most noticeably, in the pars tuberalis (PT). Several laboratories confirmed that the PT expressed melatonin binding sites in several species [5, 6], including sheep [7].

Different melatonin target sites regulate different rhythms. Strong evidence indicates that the melatonin target site controlling seasonal reproduction is in the brain [8, 9]. In contrast, the seasonal change in pelage growth that is driven by the seasonal prolactin cycle [10, 11] is regulated outside the brain. Studies on sheep have provided strong evidence that melatonin acts on the PT, and not the brain, to regulate seasonal prolactin secretion [12, 13]. Studies in hamsters bearing hypothalamic lesions showed that reproductive responses to changes in photoperiod were lost, but photoperiod-dependent rhythms in prolactin release persisted [14]. These studies form the basis of the ‘dual site’ hypothesis, which postulates that

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melatonin acts in the hypothalamus to drive reproductive rhythms but in the PT to regulate prolactin rhythms.

It is not known how the PT regulates the seasonal prolactin rhythm. In several rodent species there is evidence that thyroid-stimulating hormone (TSH) is produced by the PT [15–17], and recent research on birds [18] suggests that TSH may play a central role in photoperiod-driven processes in this class of vertebrates. In sheep, apart from gonadotropes confined to the ventral aspect [19–21], most PT cells are chromophobic and account for 90–95% of all PT cells [21]. Indeed, the phenotypic identity of the majority of cells in the PT of all species is unknown [19, 20, 22, 23]. This has led to speculation that the PT secretes a novel peptide, called 'tuberalin' [23–25]. However, as noted recently, 'the identity of tuberalin remains elusive and to date no candidate molecules have been identified' [26]. Several researchers have listed specific criteria that tuberalin should fulfill. It should be (1) evolutionarily conserved [26, 27], (2) a peptide [22, 24], (3) approximately 1 kDa [28] and (4) present in the pars distalis (PD) [29, 30]. The present report describes an initial finding that the PT contains an enriched population of substance P (SP)-immunoreactive cells. It is noteworthy that SP, which stimulates prolactin release in rodents [for a review, see ref. 31], meets all of the criteria for the elusive tuberalin.

Materials and Methods

Animal Management and Nutritional Treatment

Non-breeding season (June) Rambouillet X Columbia rams (n = 6) were injected intravenously with 25,000 IU heparin and killed with an anesthetic overdose. Animals were decapitated and perfused through the carotid arteries with 1 liter 1% sodium nitrite in 0.9% NaCl, then a 3-liter solution of cold 4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer (pH 7.4) and 1 liter 20% sucrose in 0.1 M phosphate buffer. The hypothalamus-PT complex and pituitaries were placed in the sucrose solution (for 24 h, at 4 °C), embedded in Tissue-Tek OCT (Miles Inc., Elkhart, N.J., USA) and frozen in liquid N_2-cooled isopentane. Sections (20 μm) were mounted onto Silane-coated slides and kept at −80 °C. Procedures were approved by the University of Wyoming Animal Care Committee (IACUC No. A-3126-01).

Immunocytochemistry

A series of sections was washed in 0.01 M phosphate-buffered saline (pH 7.4; all washes 3 × 5 min at room temperature). Sections were incubated (for 72 h at 4 °C in a humidified chamber) in phosphate-buffered saline containing 1% Triton, 10% normal goat serum and a guinea pig SP antibody (T5019; Peninsula Laboratories, San Carlos, Calif., USA; 1:1,000) and, for dual labeling, a rabbit anti-ovine β-luteinizing hormone (βLH) antibody (1:10,000; AFP697071P, National Institute of Diabetes and Digestive and Kidney Diseases). Slides were washed, incubated in Texas Red goat anti-guinea pig IgG and biotinylated goat anti-rabbit (both Jackson Laboratories, West Grove, Pa., USA; 1:200, for 1 h at room temperature), washed, placed in FITC-streptavidin (Vector, Burlingame, Calif., USA; 1:200, for 1 h at room temperature), washed and mounted with Vectashield with DAPI (Vector). Pre-absorbing the SP antibody with 10 μg/ml SP (American Peptide Co., Sunnyvale, Calif., USA) resulted in no staining (data not shown). This antibody did not crossreact with 10 μg/ml neurokinin B. Additional controls included omission of the primary antibodies, and incubation in the primary antibodies followed by incubation with secondary antibodies raised in inappropriate species.

The percentage of immunoreactive cells relative to the total number of DAPI-labeled cells was calculated. As the PT is densely vascularized (fig. 1), it is not useful to express cell

density relative to area. It is noteworthy that no SP-immunoreactive cells were evident in 1 animal, although βLH was identified; the ram was excluded from analysis. Data were statistically compared using Student's paired t test and are presented as mean ± SEM.

Reverse Transcription PCR

RT-PCR was performed as previously described [32]. After the first-strand cDNA synthesis, samples were amplified for preprotachykinin A. Since the ovine preprotachykinin A mRNA sequence was not available from data bases, primers were identical to those previously described by others [33] for the bovine sequence (P01289). The sense primer, spanning exon 1 and exon 2, started at position 123 of the published nucleotide sequence, and the antisense primer, in exon 3, ends at position 323 (sense, GGGTCAGCTGCGAAATCC; antisense, CTTGGGTCTCCGAGCGATTACT). This fragment is present in all splice variants (α, β, γ and δ) of preprotachykinin A mRNA. Samples were visualized using a 0.7% agarose gel with ethidium bromide.

Results

SP-immunoreactive cells were abundant in the PT (58.1 ± 17.3%; fig. 1A–F) and sparse in the PD (0.9 ± 0.3%; $p < 0.01$; fig. 1G). These PT tachykinin-immunoreactive cells were often in close association with the hypophyseal vessels of the primary portal plexus that course through this pituitary subdivision (fig. 1). Many PT cells are characterized by an extremely thin cytoplasmic area relative to the nucleus, and these cells were often labeled for SP. No evidence of SP colocalization with βLH was detected, even in the ventral PT where gonadotropes are abundant (fig. 1A, B, D). RT-PCR for preprotachykinin A mRNA revealed a clear distinct band at the expected product size of approximately 200 base pairs (fig. 1H). This band was absent when PCR was performed without RT.

Discussion

The present study provides compelling evidence that SP is abundant in the ovine PT, whereas in the PD, comparatively few cells are immunoreactive for this tachykinin. This study concurs with our earlier investigation [34] in the ovine fetus that the PD contains few SP-immunoreactive cells. We have previously reported [35] how the PT, as the zona tuberalis, extends ventrally down the anterior face of the adenohypophysis, projecting 'fingers' into the PD. Thus, we cannot discount the possibility that these PD tachykinin-immunoreactive cells are of PT origin.

For the PT to produce a factor, which then acts on the lactotropes, the PT must release this factor into either the (1) hypophyseal portal system or (2) extracellular fluid, from which it then moves through the interstitial spaces to the PD. Only 2 preliminary studies have investigated whether tachykinins are released into the hypophyseal portal blood. In a preliminary investigation in the rat, SP levels increased in the hypophyseal portal blood relative to jugular blood following hypoxia, but data were variable [36]. It has been proposed that SP is not released into the hypophyseal portal system of the ewe [37]. Some caution is due as blood samples were collected during the breeding season. If SP is driving prolactin release, then it will be at its nadir during the breeding season in sheep. To emphasize this point, if gonadotropin-releasing hormone concentrations were analyzed in samples harvested sporadically during anestrus, over 90% would be below the sensitivity of the assay. There are no studies determining if a PT factor could be released in the extracellular fluid to act through a paracrine pathway.

SP acts preferentially through the NK-1 receptor but also the NK-2 receptor, with lower affinity for the NK-3 receptor [38]. SP binding has been shown on lactotropes in the rat [39].

Studies on rodents consistently report that brief (3–10 min) SP exposure stimulates prolactin secretion [for a review, see ref. 31]. In long-term ovariectomized ewes, a single 70-nmol intravenous SP injection had no effect on prolactin release [40]. These ewes were long-term ovariectomized with no estrogen replacement. The induction of NK-1 receptors is estrogen dependent [41], and there is evidence that tachykinins may not affect prolactin release in the ovariectomized rat [42]. Second, SP is rapidly degraded [37], and a 70-nmol dose may not have been sufficient to raise SP levels in the hypophyseal portal system. Indeed, SP administered directly into the hypophyseal portal circulation of the rat powerfully stimulated prolactin release, but when the same dose was administered intravenously, there was no effect [43]. Analysis of the rodent studies [43–49] suggests that SP concentrations must exceed 100 nM at the level of the pituitary gland to evoke a prolactin response. It is not known if this dose is physiological. An insufficient stimulus to lactotropes may also have been provided by SP infusion (1.5 pmol/kg/min) in men [50].

The PT is a distinct anatomical subdivision of the pituitary gland that remains an enigma. It is clearly an integrative site expressing high densities of insulin-like growth factor 1 [51], leptin [52] and melatonin receptors that potentially serve to modulate neuroendocrine activity. Although βTSH mRNA has been reported in the ovine PT, the βTSH protein has not yet been detected here [19, 21]. βTSH protein may be synthesized and released extremely rapidly, rendering it undetectable by our approach. Moreover, Hanon et al. [53] recently argued that ‘PT-derived TSH does not act as the pituitary paracrine signal between melatonin and prolactin secretion’. The present study provides evidence that the PT is an enriched source of SP. It is noteworthy that at least 3 other tachykinins are derived from the preprotachykinin A mRNA: neurokinin A, neuropeptide-γ and neuropeptide K may also be synthesized; neuropeptide K powerfully augments the SP-induced salivary response [54]. As these tachykinins may be coreleased, there are surprisingly few studies on the effect of 2 or more tachykinins on any system. Clearly, more research is required to establish whether PT-derived tachykinins play a role in seasonal hormone rhythms.

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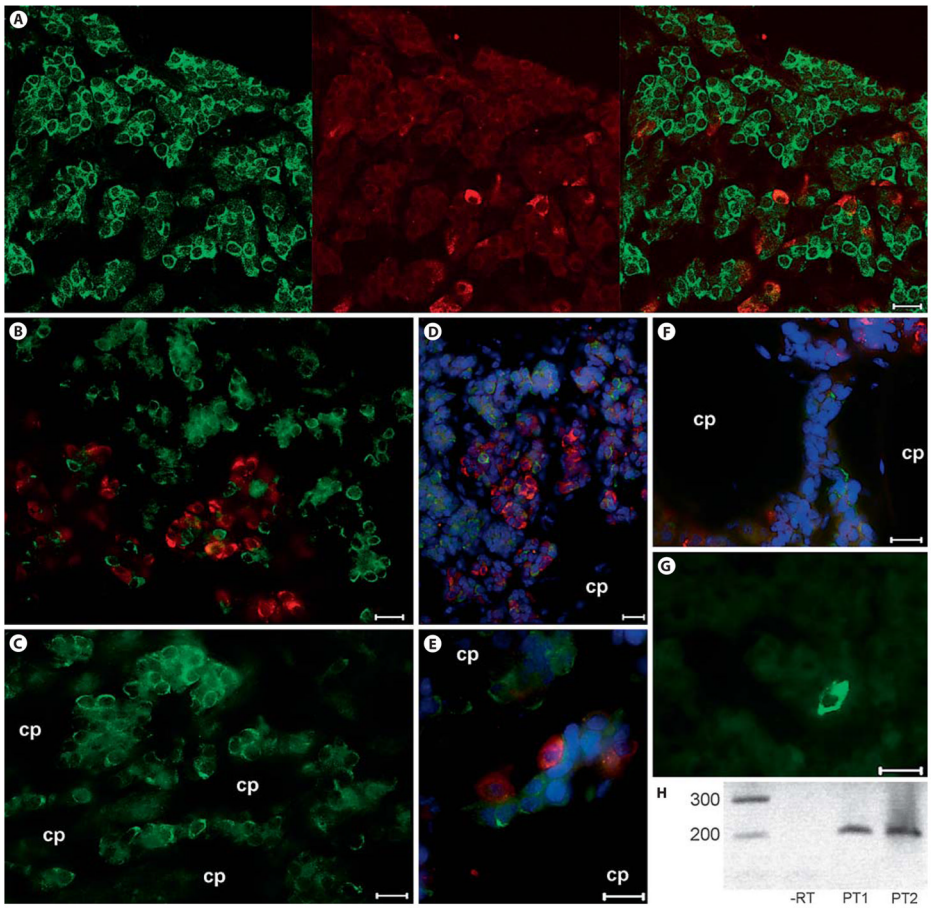

Fig. 1.

Photomicrographs and RT-PCR showing the presence of SP in the ovine PT. **A**, **B**, **E** βLH (red) and SP (green) do not colocalize in gonadotropes of the ram pituitary. **C–F** These SP-immunoreactive cells were frequently observed to surround hypophyseal portal capillaries. **G** Very few SP-immunoreactive cells were evident in the PD. **H** RT-PCR revealed distinct preprotachykinin A mRNA in 2 different sheep. If no RT was performed, no band was evident. Red = βLH; green = SP; blue = DAPI; cp = capillary. Scale bar = 10 μm.