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# Tachykinins and their gene expression in the anterior pituitary of the siberian hamster – effects of photoperiod, thyroid hormones, and analogs of hypothalamic hormones

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**The anterior pituitary gland of the Siberian hamster contains high concentrations of tachykinins compared to other laboratory rodents. In this investigation we studied the relative quantities of neurokinin A (NKA), neuropeptide gamma (NPG), and neuropeptide K (NPK) present in extracts of anterior pituitaries from this species. The anterior pituitary extracts, purified by HPLC, contained similar quantities of NKA and NPG, and lower quantities of NPK. The anterior pituitary gland of the Siberian hamster contained mRNA encoding β-preprotachykinin A, which is a precursor of substance P, NKA, and NPK. This fact proves that the anterior pituitary gland of the Siberian hamster has the ability to synthesize tachykinins. Animals exposed to short photoperiods had higher concentrations of tachykinins in the pituitary gland, and triiodothyronine markedly depressed the stores of these peptides in the anterior pituitary. In some groups of animals, the somatostatin analog octreotide induced a small, but significant decrease of the levels of NKA in the pituitary. The present results, together with previously published findings, indicate that thyroid hormones and estrogens are the most active endogenous substances to suppress the levels of anterior pituitary tachykinins in the Siberian hamster.**

**Keywords:** tachykinins; Siberian hamster; anterior pituitary; neurokinin A, substance P

## Introduction

There is increasing evidence that tachykinins can influence the secretion of several anterior pituitary (AP) hormones (Jessop *et al.*, 1992). Substance P (SP) and neurokinin A (NKA) have been demonstrated in the AP gland of the rat, although at concentrations considerably lower than in the hypothalamus (Brown *et al.*, 1990; Coslovsky *et al.*, 1984; Debeljuk *et al.*, 1992b; Villanúa *et al.*, 1992). Another tachykinin, neuropeptide K (NPK), however, could not be demonstrated by HPLC in the AP gland (Brown *et al.*, 1990). Tachykinins are probably synthesized in the AP gland,since tachykinin gene is expressed in the rat AP (Brown *et al.*, 1990; Jonassen *et al.*, 1987).

We have recently demonstrated that the AP of the Siberian hamster contains markedly higher levels of tachykinins than the same gland of other laboratory rodents such as the rat, golden hamster, and mouse (Debeljuk & Bartke, 1994). The reason for these high tachykinin levels in the AP in this species is not apparent. Therefore we felt that it would be of interest to elucidate, by HPLC separation, the tachykinin profile present in the AP of the Siberian hamster. This is particularly important, since most, if not all, of the existing antisera to NKA have cross-reactivity with NPK and/or neuropeptide gamma (NPG), because these two peptides contain the complete sequence of NKA. This means that NPK and NPG are detected in the radioimmunoassay for NKA. By HPLC these peptides can be separated and therefore their relative levels can be determined. In addition, due to the high tachykinin content of the AP of the Siberian hamster it is important to know if these tachykinins can be actually synthesized in the AP, by demonstrating tachykinin gene expression in the RNA extracted from this gland.

We already know that estradiol is an important regulator of the AP tachykinin stores (Brown *et al.*, 1990; Coslovsky *et al.*, 1984; Debeljuk *et al.*, 1992b), but other factors or hypothalamic hormones may also regulate the presence of tachykinins in the pituitary cells. Therefore, we decided to treat Siberian hamsters with substances that depress the function of a particular type of pituitary cell and to correlate their effects with the concentrations of tachykinins. The rationale for these experiments was based on the speculation that if a substance depressed the function of a cell that also produces tachykinins, tachykinin concentrations should fall. In this investigation we used female Siberian hamsters kept either under long (LD) or short (SD) photoperiod. This species is particularly sensitive to environmental light, and exposure to short photoperiod effectively induces gonadal regression (Yellon & Godman, 1987). We have previously observed that the AP from Siberian hamsters kept under SD has significantly higher tachykinin concentrations than the AP from animals kept under LD (Debeljuk & Bartke, 1994). Therefore, we considered it of interest to test the same substances also in this animal model.

## Results

### *HPLC*

The purification of the AP extracts, followed by radioimmunoassay for NKA-immunoreactive substances resulted in the detection of three peaks: the first one, which showed a subpeak, corresponds to NKA, which eluted at tube numbers 30–32. A second peak, which appeared in tubes 43–46, corresponds to the elution of NPG, and finally, a smaller, but evident peak, was detected between tubes 60–63, and corresponds to the elution of NPK (Figure 1).

### *Tachykinin gene expression in AP and hypothalamic extracts*

The β-preprotachykinin A (β-PPT) gene was expressed in all the experimental samples, hypothalami and anterior pituitaries from intact and ovariectomized Siberian hamsters (Figure 2). No expression was detected in the liver of the same animals, which was used as negative control. The mRNA expression of β-PPT did not exhibit marked differences in the expression pattern of the hypothalamic and anterior pituitary samples. The gene expression studies in this species provide evidence for β-PPT mRNA presence both in the hypothalamus and AP.

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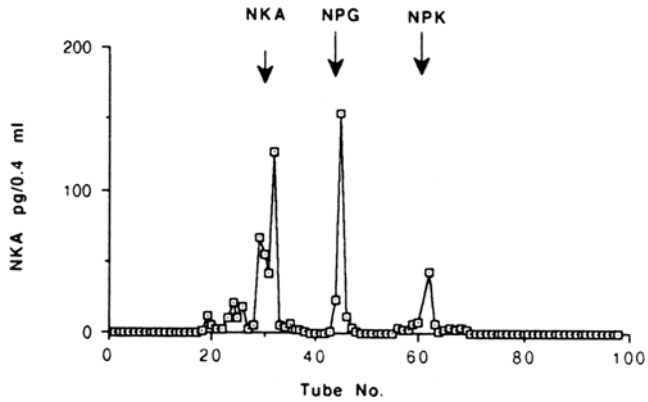


**Figure 1** Elution profile of an anterior pituitary extract from Siberian hamsters. The position of migration of NKA, NPG and NPK is shown by the respective arrows

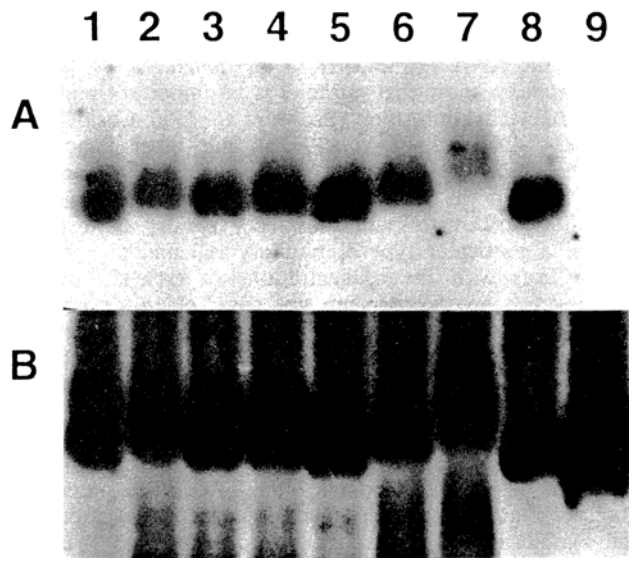


**Figure 2** Panel A: Northern blot analysis of β-preprotachykinin A gene expression in the anterior pituitary and hypothalamus of intact and ovariectomized Siberian hamsters. 1 AP from intact animals, 2 AP from ovariectomized animals, 3 Hypothalamus from intact animals, 4 hypothalamus from ovariectomized animals, 5 AP from intact animals 6 AP from ovariectomized animals, 7 Hypothalamus from intact animals, 8 AP from intact animals, 9 Liver; Panel B: After hybridization, the blots were normalized against the leels of 18s bovine ribosomal RNA probe. The number of the samples correspond to those of panel A

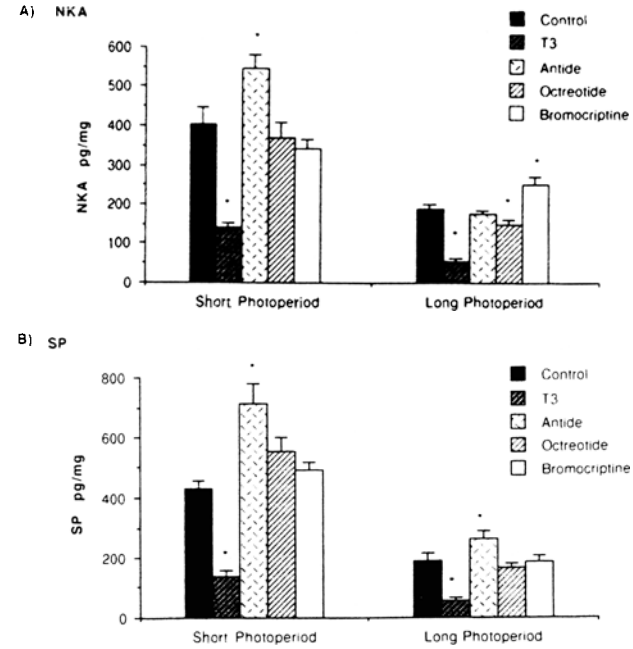


**Figure 3** Effect of triiodothyronine (T3), octreotide, Antide and bromocriptine mesylate on the concentrations of NKA-(Panel A) and SP-(Panel B) immunoreactive peptides in Siberian hamsters under short or long photoperiods. Bars represent mean ± SE. No. of animals/group: 10–11. $*P<0.05$ *vs* control

**Table 1** Effect of ovariectomy on NKA and SP-immunoreactive substances in the anterior pituitary gland of female Siberian hamsters kept in short photoperiod

| *Treatment* | *NKA pg/mg* | *SP pg/mg* |
|---|---|---|
| Intact (9) | 443.58 ± 36.44 | 473.4 ± 48.99 |
| Ovariectomy (10) | 523.54 ± 42.62 | 569.3 ± 50.05 |
| | NS | NS |

Values are Means ± SE. No. of animals in parentheses. NS: not significant

*Effect of different treatments on the tachykinins in the AP*

Vehicle-injected hamsters exposed to SD had significantly higher SP and NKA concentrations than the animals exposed to LD (Figure 3). In SD-exposed animals, T3 was highly effective in reducing both NKA and SP concentrations in the AP (Figure 3A and B) ($F\ 4,43 = 19.19$, $P<0.05$; $F\ 4,41 = 27.49$, $P<0.05$, respectively). Antide, on the other hand significantly increased both NKA and SP concentrations in the AP. In an additional experiment, however, ovariectomy, which also suppresses estradiol output, did not significantly affect AP tachykinin concentrations in SD-exposed animals (Table 1). Octreotide and bromocriptine did not induce significant changes in AP tachykinin concentrations of SD-exposed Siberian hamsters.

In LD-exposed hamsters, T3 was again highly effective in reducing both tachykinins in the AP (Figure 3A and B) ($F\ 4,40 = 25.93$, $P<0.05$ for NKA; $F\ 4,39 = 9.84$, $P<0.05$, for SP). Antide did not significantly alter NKA concentration, although it induced a small, but significant increase of SP concentration ($P<0.05$). Octreotide induced small decreases in tachykinin concentrations, which were significant in the case of NKA ($P<0.05$). Treatment with bromocriptine did not alter SP concentration but induced a significant increase of NKA concentration in the AP ($P<0.05$).

## Discussion

The results of this investigation show that the AP of the Siberian hamsters contains, in addition to SP, high levels of NKA, NPG, as well as relatively lower amounts of NPK, as revealed by HPLC separation. Previous studies had failed to reveal the presence of NPK in the AP of the rat (Brown *et al.*, 1990), but since the β-PPT gene is expressed in the rat AP. NPK is most likely synthesized in the gland. The failure to detect NPK in previous reports may have been due to low relative content of this peptide, or to quick proteolytic conversion to NKA.

We have recently confirmed the presence of NPK in the AP of the Siberian hamster by RIA using a highly specific antiserum to NPK, developed in our laboratory. This antiserum recognizes only the non-NKA portion of its molecule, and therefore does not cross-react with NKA or

NPG (unpublished results). Using this very specific anti-NPK serum in a radioimmunoassay system we have found that the amount of NPK present in the AP of the Siberian hamster is actually higher than that suggested by the modest NPK peak in the present investigation. Since the anti-NKA serum used in the present investigation has about 60% cross-reactivity with NPK, it may have underestimated the actual amount of the latter peptide.

The strong β-PPT mRNA expression in the AP of Siberian hamsters furnishes evidence that the high amounts of tachykinins present in this species are most likely synthesized within the gland. It is not possible at the present time to ascertain the role that these relatively high stores of tachykinins may have in this species, as compared with other rodents with much lower tachykinin stores in the AP, such as the rat or the Syrian hamster. One may feel tempted to relate to these high levels to the ability of regulating gonadotropin and prolactin secretion in response to the decrease of environmental lighting conditions. However, this ability is shared by the golden hamster, a species with much lower tachykinin concentrations in the AP. Therefore, the function of the tachykinins in the Siberian hamster may have some special importance in the control of AP function, although this has yet to be clearly elucidated.

These studies confirm our previous report that the AP from SD-exposed Siberian hamsters has higher tachykinin concentrations than the AP from LD-exposed animals (Debeljuk & Bartke, 1994). This increase in tachykinin stores is most likely due, in part, to a decrease of circulating estrogens in SD hamsters, since these animals undergo gonadal regression. Estradiol has been previously demonstrated to be a potent influence to decrease AP tachykinin concentrations in the rat and in the Siberian hamster (Brown *et al.*, 1990; Coslovsky *et al.*, 1984; Debeljuk & Bartke, 1994; Debeljuk *et al.*, 1992b). The other factor that seems to be a major influence on the tachykinin stores in the AP is T3, which in our present studies was shown to be as active in LD- as in SD-exposed animals. Other authors had previously reported that in the rat, thyroid hormones induced a significant decrease of SP concentrations in the AP (Jonassen *et al.*, 1987). This effect is present in the Siberian hamster as well, as shown in the present investigation, and this suggests that the thyrotrophs may be a major source of tachykinins in the AP. Furthermore, in rats and golden hamsters the pineal gland has been shown to depress thyroid function (Vriend, 1981). In golden hamsters exposed to SD the thyroid function was found to be significantly depressed (Vriend, 1981). If this is also the case in the Siberian hamster, then the depression of thyroid function in animals kept in SD is most likely to be the second factor, in addition to the decrease of estrogens, responsible for the increase of tachykinins in the AP of SD-exposed Siberian hamsters. In the rat and in the human AP it was previously demonstrated that a subset of thyrotrophs contain tachykinins (Brown *et al.*, 1991; Roth & Krause, 1990). The same reports, however, show that some somatotrophs also contain tachykinins. In the present investigation, the somatostatin analog octreotide has been only marginally effective in decreasing NKA-immunoreactive peptides, with its apparent effect reaching statistical significance only in LD-exposed hamsters. This fact suggests that somatotrophs may contribute somewhat to the whole tachykinin content of the AP, but this contribution is far less important than the thyrotrophs.

The LH-RH antagonist Antide, on the contrary, was shown to induce increases in AP tachykinin concentrations, but this effect may be indirect, through a suppression of ovarian estradiol secretion. Antide was effective in this respect also in SD-exposed hamsters, which already have gonadal suppression. One explanation for this puzzling effect could be that some, although decreased, quantities of estrogens could be still secreted by the ovaries of these animals, and the treatment with Antide may have further decreased circulating estradiol levels, therefore increasing AP tachykinin concentrations even more. In order to shed some light to the possible mechanism of this effect in SD hamsters, we included an additional experiment, performing ovariectomies in animals previously exposed to SD. Ovariectomy eliminated any vestige of estrogen production by the ovaries, but it failed to further elevate AP tachykinin concentrations, although in both, SP and NKA, there was a slight increase. In LD-exposed hamsters, we had previously demonstrated that ovariectomy is followed by a marked increase in the AP tachykinin concentrations (Debeljuk & Bartke, 1994). Taken together, these results in SD-exposed animals indicate the possibility that Antide may have some direct effect on the AP. Finally, the suppression of mammotroph function does not seem to be of quantitative importance concerning AP tachykinins, since the treatment with the dopamine agonist bromocriptine did not decrease SP or NKA in the AP, and in fact, it increased NKA concentrations in LD-exposed animals.

In conclusion, the AP of the Siberian hamster contains high levels of tachykinins, including SP, NKA, NPG, and to a lesser extent also NPK, which is definitely present in the gland of this species. These tachykinins are most likely synthesized in the AP gland, since it expresses the β-PPT gene, and their concentrations are regulated mainly by estrogens and thyroid hormones.

## Materials and methods

### *Animals*

Siberian hamsters were bred and raised in our vivarium, were fed laboratory chow and had free access to tap water. Young adult female animals were used throughout the investigation. They were kept either in LD (16 h light–8 h darkness) or in SD (6 h light–18 h darkness) photoperiods. Female hamsters in SD exhibit vaginal cytology characteristic of diestrus. However, in our colony, the females in LD are most of the time in a state of prolonged diestrus if, as was the case in these experiments, no male was allowed in the same cage.

### *HPLC studies*

Anterior pituitary glands from young adult Siberian hamsters were prepared by pooling about 15 glands that were quickly removed after the decapitation of the animals and immersed in ice-cold 2N acetic acid. The glands were homogenized by sonication, centrifuged, and the supernatant aspirated and lyophilized. Immediately before the injection into the HPLC apparatus, the extract was dissolved in 20% acetonitrile in 0.1% trifluoroacetic acid-water solution, and centrifuged to eliminate insoluble particles. The supernatant was injected onto a C18 RP-HPLC column (Beckman, Ultrasphere Octyl 5 μ; 4.6 cm × 25 cm) equilibrated with the same solvent. The column was eluted with a linear gradient of 0.5% acetonitrile/min at a low rate of 1 ml/min. Fractions were collected at one min intervals and lyophilized for subsequent tachykinin determinations. Following each injection of extract or pure peptides, the column was extensively washed with 20% acetonitrile-0.1% trifluoroacetic acid.

### *Tachykinin gene expression in the AP and hypothalamus of Siberian hamsters*

A 560 bp cDNA inserted in PGEM-T encoding β-PPT was kindly provided by Dr J. Krause (Department of Anatomy and Neurobiology, Washington University, St. Louis, MO). The cDNA was propagated by transforming into XL-1 Blue bacterial strain (Promega Corporation, Madison, WI) and linearized with BamH1. Digoxigenin labeled βPPT cRNA was prepared using SP6 RNA polymerase, as per RNA-labeling protocol (Boehringer-Mannheim, Indianapolis, IN). Total RNA was isolated from hypothalamus and anterior

pituitary of intact or ovariectomized (30 days ovariectomy) female Siberian hamsters using guanidinium acid-thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi, 1987). Total RNA was quantitated by measuring the spectrophotometric absorbance of samples at 260 nm. Ten μg aliquots of total RNA were size fractionated on 1.5% agarose gels. The RNA was transferred onto a positively charged nylon membrane (Micron Separation, Inc., Westboro, MA). The membranes were hybridized with the hybridization buffer containing DIG-labeled cRNA probe overnight at 63°C. The membranes were then incubated with antidigoxigenin-alkaline phosphatase antibody (1:5000) in blocking solution for 30 min at room temperature with gentle agitation. Subsequently, the membranes were washed twice with maleate buffer for a total of 30 min at room temperature to remove the unbound antibody. The membranes were then equilibrated in Genius buffer 3 (100 mM Tris-HCl NS 100 mM NaCl, pH 9.5 containing 50 mM MgCl2) for 3–5 min. The individual membranes were placed between plastic sheet protectors to which 500 ml of diluted chemiluminescent substrate (Lumigen PPD; Genius buffer 3: Lumigen PPD 1:100) were added to the surface of the membrane. After 1 min the excess of chemiluminescent alkaline phosphatase substrate was drained and the top sheet of the plastic sheet protector was lowered to eliminate any air bubbles that may have been present. The membrane was sealed in a bag and exposed to Fuji Medical X-ray films for 2–3 h at room temperature to analyse the mRNA expression. Afterwards, the membrane was stripped with stripping solution and reprobed with 18S bovine ribosomal RNA to check the loading discrepancy. This same procedure was repeated in triplicate samples.

*Tachykinins in the AP of Siberian hamsters*

Two groups of female Siberian hamsters were used: in the first group, the hamsters were kept under LD, while the hamsters of the second group had been kept under SD for about two months at the time of starting the treatment. To both LD and SD hamsters the following treatments were given: (1) Control, injected with 0.1 ml of 0.01% Tween 80 (Sigma Chemical Company, St. Louis, MO); (2) Triiodothyronine (T3) (Sigma) 20 μg/day; (3) The LH-RH antagonist Antide (Bachem California, Los Angeles, CA) 25 μg/day, this dose is effective for more than 24 h, according to Ljungqvist *et al.* (1988); (4) Octreotide (Sandostatin, Sandoz Pharmaceuticals, East Hanover, NJ) 20 μg/3 time a day, this injection schedule was chosen in order to obtain sustained inhibition of serum GH (Dr L. Felarca, Sandoz Pharmaceuticals, personel communication); (5) Bromocriptine mesylate (Sandoz Pharmaceuticals) 300 μg/day. All the substances administered were dissolved or suspended in 0.01% Tween 80 and injected sc, during the morning hours. The treatments were continued for 10 days and the last injection was given 2–3 h before killing of animals. The animals were killed by decapitation, the skulls were opened and the anterior pituitaries were collected and quickly immersed in ice-cold 2N acetic acid (0.5 ml/AP). After all the AP were collected, the tubes were heated in a boiling water bath for 10 min, in order to inactivate proteolytic enzymes, homogenized by sonication, centrifuged and the supernatant was aspirated, frozen and later lyophilized.

One additional experiment, complementary to that with Antide in SD animals, to evaluate the effects of the removal of the ovaries, and therefore of any possible remnant of estrogen secreted by these gonads after regression due to exposure to SD, was performed in female Siberian hamsters that had been exposed to SD for 35 days. They were submitted to bilateral ovariectomy, performed by the dorsal route under ether anesthesia. The animals were killed 15 days after the ovariectomy, and the AP glands were processed as indicated above.

*Assays*

SP and NKA were determined by double-antibody radioimmunoassays developed in our laboratory and previously described (Debeljuk *et al.*, 1990; Debeljuk *et al.*, 1992a). Synthetic SP and NKA (Cambridge Research Biochemicals, Wilmington, DE) were used as standard preparations. Bolton-Hunter labeled $^{125}$I-SP and $^{125}$I-NKA (Amersham Corp., Arlington Heights,, IL) were used as tracers. Before performing the assays, the AP extracts were dissolved in 500 μl of assay buffer (0.5% bovine serum albumin, bacitracin 20 μM, PBS). The choice of bovine serum albumin may be critical, since some albumins apparently contain proteolytic activity, which may render the assay unsuccessful. In these assays bovine serum albumin isolated by heat shock (Sigma Chemical Co.) was used with good results. The solution was centrifuged to eliminate insoluble particles, and 125 μl (equivalent to 0.25 AP) was dispensed into each assay tube. The incubation was carried out for 5 days, in polypropylene tubes, then second antibody was added; followed by 10% polyethyleneglycol in PBS, centrifuged, the supernatant was aspirated, and the precipitate was counted in a gamma counter.

*Statistical analysis*

The significance of the differences between groups was assessed by means of ANOVA, followed by Fisher's PLSD and Dunnett's tests, using a computer program (Statview 512) for the Macintosh.

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