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Tachykinins in the Pineal Gland: Effect of Castration and Ganglionectomy

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DEBELJUK, L., A. ARCE, M. GARCIA BONACHO, A. BARTKE AND A. I. ESQUIFINO. *Tachykinins in the pineal gland: Effect of castration and ganglionectomy*. PEPTIDES **19**(6) 1073–1078, 1998.—In this investigation, the presence of NKA-immunoreactive substances was determined in pineal glands from intact, castrated and castrated, testosterone-treated male rats. The effect of environmental light, melatonin treatment and superior cervical ganglionectomy on pineal NKA-immunoreactive substances was also investigated. The results obtained show that NKA is present in measurable amounts in the rat pineal, and NPK is probably also present. Orchidectomy was followed by an increase in the content of NKA-immunoreactive substances in the pineal gland. The replacement treatment with testosterone propionate in castrated rats blocked this effect. NKA-immunoreactive substances were not significantly different quantitatively in pineals from rats killed under light or under darkness. The removal of the superior cervical ganglia was followed by a significant increase in the NKA-immunoreactive substance content in the pineal gland of male rats. These results indicate that NKA and other tachykinins are present in the pineal gland of the male rat, and they seem to be regulated by gonadal hormones and the innervation originated from the superior cervical ganglia. © 1998 Elsevier Science Inc.

NKA Tachykinins Pineal Testosterone Superior cervical ganglia Castration

THE pineal gland influences many physiological functions, among them those related to reproduction and the response to environmental photoperiod (12,16,17). Many of these pineal influences are exerted through melatonin, an indole-like substance that is synthesized by the pineal gland (16,17). The secretion of melatonin is regulated by a number of neurotransmitters, mainly released from noradrenergic terminals that originate in the superior cervical ganglia (14). Several reports have indicated that the pineal gland is also innervated by fibers that contain substance P (SP) (8,19). These SP-containing fibers do not seem to originate in the superior cervical ganglia, since their ablation does not modify the intensity of SP immunoreactivity in the pineal gland (18). Receptors for SP were demonstrated in the pineal gland (10). These findings suggest that SP may play a role in pineal function. Surprisingly, SP did not modify cAMP formation, *N*-acetyl transferase activity, or melatonin secretion by rat pineals in vitro (11,19,21).

SP is co-synthesized with at least three other tachykinins, namely neurokinin A (NKA), neuropeptide K (NPK), and neuropeptide gamma (NPG) (13). No report, however, has been published on the possible existence of these tachykinins in the pineal gland.

On the other hand, gonadal hormones were demonstrated to affect pineal function (1). Melatonin secretion was shown to be affected by estradiol and progesterone (1,15). Gonadal hormones were shown to affect norepinephrine synthesis in sympathetic nerve terminals and to modify the response of pinealocytes to adrenergic stimulation (1). Through these mechanisms gonadal steroids may be able to influence the secretion of melatonin.

In the present investigation, we have determined the presence of NKA-immunoreactive (NKA-IRS) substances in the pineal gland of male rats. Since SP and other tachykinins are in a large part cosynthesized (13), we expected to find measurable amounts of NKA in the pineal gland. In

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addition, we decided to investigate whether melatonin or gonadal steroids are able to regulate the levels of tachykinins in the pineal gland. Accordingly, the effect of castration on the content of NKA-IRS immunoreactive substances in the pineal gland was studied. It has been demonstrated that in different systems, other tachykinins may be more potent than SP in eliciting effects (12,20). It may, therefore, be possible that other tachykinins may have more consistent effects on the pineal gland, as compared with SP.

As it was mentioned before, in a previous study it was demonstrated that the removal of the superior cervical ganglia did not result in apparent morphological changes of SP immunoreactivity in the pineal gland (18), but in that investigation SP was not quantitatively measured. We then decided to investigate the effect of the superior cervical ganglionectomy on the NKA-IRS content in the pineal gland using radioimmunoassay measurements.

It is well known that most of the neurotransmitters and hormones of the pineal gland exhibit circadian variations that account for their effects on pineal function (9,16,17). Taking these data into consideration, we decided to determine the possibility that tachykinins in the pineal gland may have circadian variations.

In summary, the aims of the present study were the following:

1. To determine whether tachykinins other than SP are present in the pineal gland.
2. To investigate whether these tachykinins exhibit circadian variations in the pineal gland.
3. To investigate the possibility that gonadal hormones may modulate tachykinin content in the pineal gland.
4. To investigate whether noradrenergic fibers from the superior cervical ganglia have a modulatory influence on pineal tachykinin content.

METHOD

This investigation was carried out partly in Carbondale, IL, USA, and partly in Madrid, Spain.

In the experiments carried out in the USA, adult male rats of the Sprague–Dawley strain (Harlan Sprague–Dawley, Indianapolis, IN) were used. In the experiments carried out in Madrid, adult male rats of the Wistar strain (raised in the School of Medicine vivarium) were used. The animals were kept in quarters with controlled temperature (21°C) and light (12-h light:12-h dark, from 6 a.m. to 6 p.m. in Carbondale, IL, USA, and from 8 a.m. to 8 p.m. in Madrid, Spain), and were fed standard laboratory chow and water ad lib.

Effect of Castration and Gonadal Hormones

Four experiments were carried out in Carbondale, IL, USA. In the first experiment, 10 adult male rats were castrated using a scrotal approach, under light ether anesthesia. A group of 10 intact male rats of the same age, that were sham-operated, were considered as controls. Fifteen days after surgery, the animals were killed by decapitation and the pineal glands were quickly exposed, immediately removed and placed in tubes containing 0.5 ml of ice-cold 2 N acetic acid in water. The pineals were then heated in a boiling water bath for 10 min in order to inactivate proteolytic enzymes, homogenized by sonication, centrifuged, the supernatant was aspirated and lyophilized. The extracts were kept at −20°C until the assays were performed.

The second experiment was a replication of the first, under similar conditions. In the third experiment, male rats were castrated, using the same procedures as used in the first experiment. Ten rats were subsequently treated with testosterone propionate (TP) (Sigma Chemical Co., St. Louis, MO) (300 μg/every 2 days) and the other 10 rats were treated with the oil vehicle. Another group of 10 intact male rats was considered as control. Fifteen days after surgery the rats were killed by decapitation, the pineals were removed and processed as described before. In the fourth experiment, 10 male rats of similar age and weight as compared with those of the previous experiments, were castrated and killed 15 days after surgery, the pineals being collected as described before. A group of 9 normal intact male rats was killed at about the same time as castrated rats, during the morning hours (9 a.m. to 11 a.m.). Another group of 10 intact male rats was killed during the dark period (about 10 p.m.), and the pineals were collected as indicated above.

Effect of Ganglionectomy and Melatonin

The first experiment was carried out in Carbondale, IL, USA. Adult male rats were anesthetized with ether and the superior cervical ganglia were surgically removed. The neck region was explored and the bifurcation of the carotid artery was exposed. With the help of a dissecting microscope, and using very fine forceps, the superior cervical ganglia were pulled and removed. A group of intact male rats was sham-operated and considered as control. Fifteen days after surgery, the animals were killed by decapitation, the pineals were removed, and processed as previously described.

The second experiment was carried out in Madrid, Spain. Male rats were anesthetized with ether, and the superior cervical ganglia were removed. Control littermates were sham-operated. The sham-operated rats were divided into 2 groups: in the first group the rats were injected with melatonin (Sigma Chemical Co., St. Louis, MO; 30 μg/rat/day, dissolved in an ethanol-water solution) for 11 days, and in the second group, the rats were injected with the vehicle. The injections were given at 7 p.m., 1 h before the lights went off. The ganglionectomized rats were also injected daily with the vehicle. The day after the last injection of melatonin or the vehicle, 12 to 14 rats in each of the 3 groups were killed at 4 a.m., 8 a.m., 12 p.m., 4 p.m., 8 p.m.,

or 12 a.m. The pineals were removed and immersed in ice-cold 2 N acetic acid. Two pineals were put in each tube and they were processed as indicated above.

Assays

NKA was determined using a double-antibody radioimmunoassay developed in our laboratory and previously described (3). Synthetic NKA (Cambridge Research Biochemicals, Wilmington, DE) was used as standard and Bolton-Hunter-labeled 125I-NKA (Amersham Corp., Arlington Heights, IL) was used as a tracer. Since NPK and NPG have a 10 amino acid sequence identical to NKA, this assay also detects these two peptides. Other tachykinins showed either extremely low or no cross-reaction in this assay. The sensitivity of the assay is 0.5–1 pg NKA/tube. The results were expressed as pg NKA/pineal or NKA/2 pineals, according to the case.

To study the presence of the three peptides that are detected by the antiserum used in this radioimmunoassay, rat pineal extracts were submitted to HPLC, using the procedure previously described (5,6). The extract was injected onto a C18 RP-HPLC column (Beckman, Ultrasphere Octyl 5 μ; 4.6 cm $\times$ 25 cm) equilibrated with 20% acetonitrile in 0.1% trifluoroacetic acid in water. The column was eluted with a linear gradient of 0.5% acetonitrile/min at a flow rate of 1 ml/min. Fractions were collected at 1 min interval and lyophilized for NKA determinations. The results were expressed as pg NKA/0.5 ml of eluate.

In one experiment, SP was also determined. A double-antibody radioimmunoassay developed in our laboratory (7) was used to determine SP content in the pineal extracts. Synthetic SP (Cambridge Research Biochemicals, Wilmington, DE) was used as standard, and Bolton-Hunter-labeled 12I-SP (Amersham Corp., Arlington Heights, IL) was used as a tracer. The sensitivity of the assay is 1 pg SP/tube. The results were expressed as pg SP/pineal. The significance of the differences between groups was evaluated by Student's *t*-test or analysis of variance followed by Fisher's PLSD or Dunnett's tests, where appropriate.

RESULTS

The purification of rat pineal extracts by HPLC revealed the presence of a main immunoreactive peak, corresponding basically to the elution pattern of NKA (Fig. 1). A second major peak, which eluted much later, may correspond to NPK or a similar peptide, of higher molecular weight as compared with NKA. Although in previous experiments NPK in general eluted about tube No. 60 (5), in this particular run, pure, synthetic NPK consistently appeared between tubes 80 and 83. No apparent reasons are evident for this difference, because the methodology was the same as before, with only a replacement of the old column for a new one.

In the first experiment, the NKA-immunoreactive substances (NKA-IRS) contained in the pineal gland showed relatively low levels (Fig. 2). In castrated male rats, the content of NKA-IRS in the pineal gland was moderately, but significantly, higher than in the control rats ($p < 0.05$). In a replicate experiment, the values of NKA-IRS contents were higher than in the previous experiment. NKA-IRS content in the castrated rats showed a similar tendency to increase, but in this case, the differences between groups did not reach the 95% level of significance (Fig. 2). In the third experiment, the NKA-IRS content in the pineal gland, was found to be higher than in the previous experiments. Castrated rats had significantly higher NKA-IRS content in the pineal gland than in intact controls ($p < 0.05$) (Fig. 3). Castrated rats with testosterone replacement had pineal NKA-IRS content not significantly different from that in intact rats. Pineal content of SP-like-immunoreactive sub-

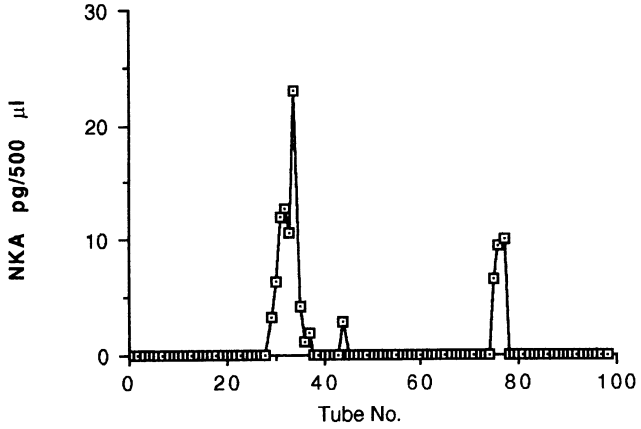

FIG. 1. Elution of NKA-IRS from rat pineal gland extracts by HPLC. Under the same conditions, pure synthetic NKA eluted between tubes 28 and 32, and NPK between tubes 80 and 83.

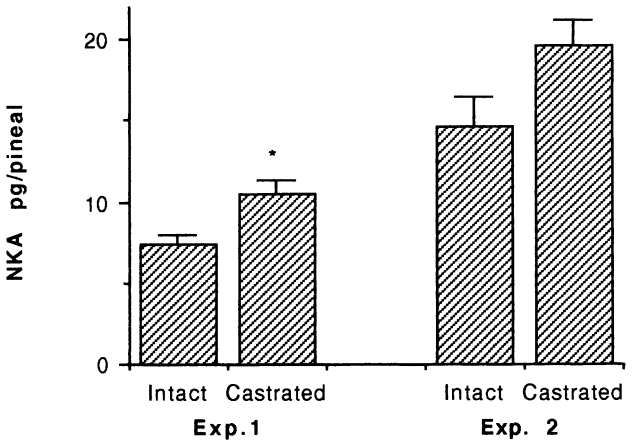

FIG. 2. NKA-IRS contents in the pineal gland of intact and castrated male rats. *$p < 0.05$

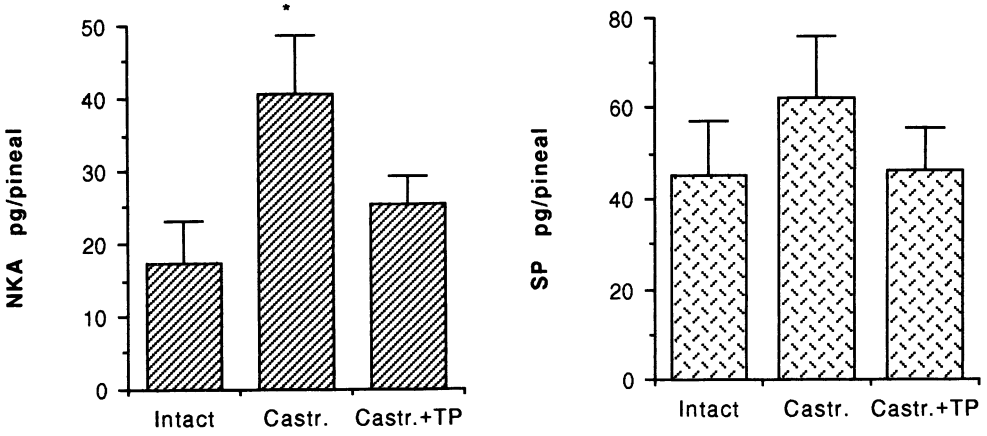

FIG. 3. NKA-IRS and SP-IRS contents in the pineal gland of intact, castrated, and castrated, testosterone-treated, male rats.*$p < 0.05$

stances (SP-IRS) followed a pattern very similar to NKA-IRS, although in this case the increase after castration did not reach the threshold for significance. In the fourth experiment, there was no significant difference between NKA-IRS content in pineals from intact rats killed during the light or dark periods (Fig. 4). The content of NKA-IRS in pineals from castrated rats was higher than in the pineals from intact rats, but in this case the differences did not reach the 95% level of significance. NKA-IRS content in pineals from rats that had been submitted to bilateral superior cervical ganglionectomy was significantly higher than in intact rats ($p < 0.05$; Fig. 5).

In the last experiment, in which Wistar rats were used, NKA-IRS contents were much lower than in Sprague–Dawley rats, and therefore it was necessary to pool the extracts of 2 pineal glands per assay tube in order to be able to get values within the range of the NKA dose-response curve. Although the pineal NKA-IRS content was slightly higher at 12 p.m. than at 12 a.m. no statistically significant differences were found at any of the times of the day investigated (Fig. 6). Intact rats treated with melatonin did not have any statistically significant differences in the pineal NKA-IRS content as compared with intact controls. Only at 12 a.m. were the values in melatonin-treated rats very close to, although not reaching, the 95% level of significance, as compared with NKA-IRS content in the pineal of intact animals. Ganglionectomized rats had no significant variations in pineal NKA-IRS content during the day. These values, however, were significantly higher than those found in intact or melatonin-treated rats at 8 a.m., 4 p.m., 8 p.m., or 12 a.m. ($p < 0.05$), but not significantly different from those in melatonin-treated rats at 12 p.m. and 4 a.m.

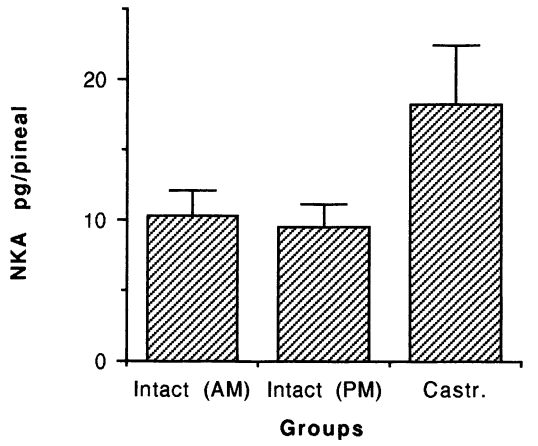

FIG. 4. NKA-IRS contents in pineal glands of intact rats killed during morning (a.m.) or evening hours (p.m.) and of castrated male rats.

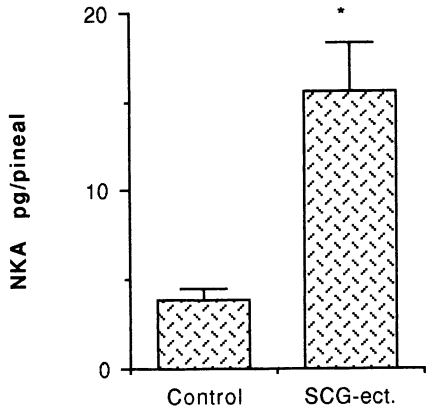

FIG. 5. NKA-IRS contents in pineal glands of intact or ganglionectomized (SCG-ect) male rats.*$p < 0.05$.

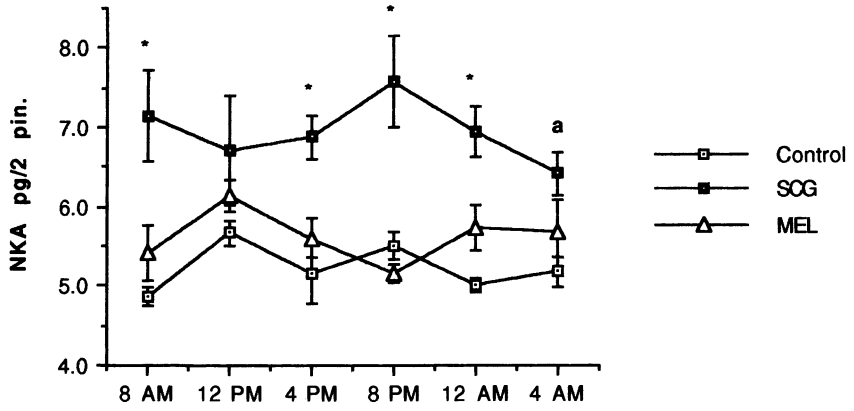

FIG. 6. NKA-IRS contents in pineal glands from intact, melatonin-treated (MEL), and ganglionectomized (SCG) male rats killed at different times of the day. $*p < 0.05$ vs. control and MEL groups; $^{a}p < 0.05$ vs. control group.

DISCUSSION

The results of this investigation show that, in addition to the previously reported presence of SP (8,10), the pineal gland also contains other tachykinins such as NKA and likely also NPK or a similar peptide. Although this is the first observation on the presence of tachykinins other than SP in the pineal gland, this finding was rather obvious, since SP is largely cosynthesized with NKA, NPK and NPG within larger peptides called preprotachykinins (13). Surprisingly, the content of NKA-IRS did not show significant modifications during the day, suggesting that tachykinins in the pineal gland do not undergo circadian variations, as it is the case with melatonin and other neurotransmitters in this gland (12,16,18). It is known that many substances contained in the pineal gland as well as hormones that affect pineal activity, show a significant circadian rhythm (9,17). The treatment with melatonin did not seem to have significantly affected NKA-IRS in the pineal gland. Therefore, it appears that environmental light does not play a fundamental role in the regulation of tachykinin stores in the pineal gland.

In a previous report (18) it was shown that the removal of the superior cervical ganglia did not affect the presence of immunoreactive SP staining in the pineal gland. This means that tachykinins are not transported into the pineal gland by fibers originated in those ganglia, raising the possibility that tachykinins may be synthesized in the pineal gland itself. If this is true, the pineal gland should contain the gene and the mRNA encoding the synthesis of preprotachykinins. We have attempted to reveal the expression of β-preprotachykinin gene in rat pineal glands using Northern blot analysis, as described in a previous report (5). So far we have not been able to obtain positive results, although this may be due to low levels of mRNAs encoding β-preprotachykinin together with a rather low sensitivity of the detection method. We plan to further study this problem using pineals from larger animals, such as pigs, which will allow us to get greater amounts of extracted mRNA. Our present investigation has confirmed the earlier finding of Ronnekleiv and Kelly (18), showing that pineal tachykinin do not originate from nervous fibers coming from the superior cervical ganglia. But our investigation further extends these previous studies by showing that, in fact, ganglionectomy is followed by an increase in tachykinin content in the pineal gland. This finding also suggests that the innervation coming from the superior cervical ganglia has actually an inhibitory influence on pineal tachykinins. Tachykinins are likely to have a regulatory role on the pineal function. However, the few studies available testing the effects of SP on pineal function have been consistently negative (11,19,21), except for one single report, which showed that SP stimulated adenylate cyclase activity in human pineal homogenates (8). In different systems, however, other tachykinins, such as NPK and NPG, have been found to be considerably more potent than SP to elicit tachykininergic effects (12,20). It is therefore possible that NPK, NKA, or NPG may exert effects on the pineal function that could be quantitatively or qualitatively different from those of SP, but this is a matter of future research.

Gonadal hormones seem to have a modulatory effect on pineal tachykinins. Although in some experiments the postcastration increase of pineal tachykinins did not reach the generally accepted threshold level of significance, the tendency toward an increase was always quite clear. In some experiments we found considerable variation in the individual values and this may have been a reason for the nonsignificant values. These findings suggest an inhibitory effect of testosterone on pineal tachykinins. In previous studies in the rat and mouse, it was demonstrated that tachykinin concentrations in the hypothalamus and anterior

pituitary gland were increased after the treatment with testosterone (3,4). Orchidectomy, on the other hand, resulted in decreased tachykinin concentrations in the hypothalamus and anterior pituitary gland (3,4) It is interesting, therefore, to observe that the effects of testosterone in the hypothalamus and pineal gland seem to be exerted in an opposite direction.

In summary, we have demonstrated that, in addition to SP, NKA is also present in the rat pineal gland, although it remains to be elucidated if these tachykinins are synthesized in situ or are transported from other neural structures. The content of tachykinins in the pineal gland seems to be modulated by androgens and also by the innervation originated from the superior cervical ganglia. It is surprising, however, that no circadian variations were detected, as the activity of the innervating fibers from these ganglia undergo changes related to the environmental light. It may be very interesting to reinvestigate these points using pineals from larger animals, whose pineal tachykinin content may be much higher and therefore possible variations may be more easily detected.

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