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Peptides 23 (2002) 1617–1623

PEPTIDES

Developmental pattern of tachykinins during aging in several organs: effect of exogenous melatonin

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Received 7 January 2002; accepted 30 April 2002

Abstract

Mammalian neurokinin A (NKA) and substance P (SP) are neuropeptides widely distributed in the body; they are potential regulators of the basal blood flow and therefore of the function of many organs and tissues. In the present investigation, we studied the age-dependent changes in NKA and SP in ovary, liver, pancreas and spleen as well as the role of exogenous melatonin on these changes. Female rats of 5, 15 or 25 months of age were studied. In the ovary, NKA concentrations did not change during aging. SP concentrations in the control group were significantly higher ($P < 0.01$) in old rats than in the other two age groups studied. Melatonin treatment resulted in reduced concentrations as compared with those of the control old rats. In the pancreas, NKA and SP concentrations increased during aging, the young rats showing significantly lower values ($P < 0.01$) than middle-aged and old rats for NKA and significantly lower ($P < 0.01$) than the old rats for SP. After melatonin treatment the differences in NKA concentrations disappeared and SP decreased in middle-aged as compared with those in old rats. In the liver, NKA and SP concentrations in the control and melatonin-treated groups did not differ significantly for the three age groups studied. Splenic NKA in control and melatonin-treated groups increased from young to middle-age up to old ages. SP concentrations showed similar values at all ages except in melatonin-treated old rats; in these animals there were significantly higher concentrations than in young melatonin-treated rats. The effect of melatonin was mainly observed on the ovary and pancreas in old rats, with a reduction in the concentrations as compared with those observed in the young groups.

Keywords: NKA; SP; Aging; Melatonin; Ovary; Pancreas; Liver; Spleen

1. Introduction

Mammalian tachykinins (TKs), neurokinin A (NKA), and substance P (SP) are neuropeptides widely distributed in the CNS and other organs. The primary function of these peptides are not completely understood [30]. Through their effects on the vascular tone, they are potential regulators of the basal blood flow and therefore of the function of many organs and tissues [7]. It has been suggested that neurokinins may play an important role in the regulation of autonomic nervous system [34] as well as in modulation of the immune system [37]. SP, among other peptides, may contribute to the local vascular control in the ovary and there may be a relationship between ovulation and vascular endothelial function [44]. Neuropeptides are also particularly important in the coordination of pancreatic exocrine and endocrine secretions. Calcitonin gene-related peptide (CGRP), inmunoreactive neurons and CGRP/SP TKs immunoreactive fibers regulate hepatobiliary activity, including hemodynamic functions of the hepatic vasculature [20]. In addition, binding sites for SP and NKA neuropeptides have been localized in primary afferent nerves which innervate several immune organs including the thymus, spleen, and lymph nodes [16].

Little is known, however, of the developmental pattern of TKs in these organs. Few studies have addressed age-related changes in TKs in non-endocrine tissues, and data on the function and changes in neurokinins during the physiological aging process are limited and the results are conflicting. Reportedly, aging of the CNS has been associated with widespread changes in TK gene expression, suggesting that alterations in tachyninergic system may have implications for the physiopathology of the diseases of the elderly [35].

Aging of the nervous system has not been associated with widespread changes in TK binding but differences in behavioral responses to TK agonists may reflect changes in other transmitter systems which respond to TK input [43]. Finally, in the immunohistochemical study of the brain of young (5 months) versus middle-aged (15 months) and old rats (23–25 months) there was no statistically significant change in

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PII: S0196-9781(02)00099-2

neuronal number and size of the neurokinin B and neurokinin-3 receptor staining [27]. The numbers of SP-LI perikarya in the central nervous system of aged hamsters at both day- and night-time were augmented three- to four-fold when compared to adults animals [40]. Recent studies from our laboratory have shown that during aging of the rats, concentrations of TKs in brain areas undergo important alterations, showing increased NKA and SP concentrations in hypothalamus of old rats in comparison to young or middle-aged rats and an opposite developmental pattern in pineal and striatum in which TK concentrations decreased with increasing age [18]. Melatonin treatment restored TK concentrations in hypothalamus of old rats showing values similar to those found in young rats. Striatal or pineal TK concentrations were not modified.

As in blood, levels of melatonin in every other fluid, tissue, and cell thus far studied are higher during the night than during the day [38]. Whereas the 24-h rhythm of melatonin is robust in young animals and humans, the cycle deteriorates during aging and may be totally abolished in neurodegenerative diseases [4,41]. In the present study we investigated the effects of aging on the concentration of NKA and SP in different body organs. Since melatonin treatment has known [17,23] to have a beneficial effect in a variety experimental neurodegenerative processes. The indication is that exogenous melatonin may delay the aging process of tissues by means of its free radical scavenging effects [21,31]. Herein we investigated the possibility that melatonin may also influence TK concentrations in different body organs during aging.

2. Material and methods

2.1. Animals and treatments

Young (3 months) ($N = 49$), middle-aged (13 months) ($N = 29$) and old (23 months) female Wistar rats ($N = 32$) from our colony were used. Animals were housed under 12 h light/dark cycles (lights on at 08:00 h) at a room temperature of approximately 23 °C. Standard rat chow and tap water were available ad libitum. The rats were divided into two groups: control and melatonin-treated. Young–control ($N = 26$), young–MEL ($N = 23$), middle-aged–control ($N = 15$), middle-aged–MEL ($N = 14$), old–control ($N = 15$) and old–MEL ($N = 17$).

2.2. Melatonin treatment

Previous data [22] testing the in vivo melatonin effect; 3 mg/kg, in young and old (24 months) albino rats had been used, yet even with such large doses, adverse side effects were not observed. In our study the effect of melatonin was studied with the dose of 150 mg/100 g BW. Melatonin (M-5250, Sigma, St. Louis, MO) was dissolved in a small volume of absolute ethanol (0.02 ml) and diluted in 0.9% NaCl up to a stock solution of 5 mg/5 ml. Melatonin injections were given at the end of the light phase, 18:30–19:00 h for 2 months. Control animals received placebo solution (absolute ethanol in 0.9% NaCl). Following the treatments the rats were decapitated at the first diestrus.

2.3. Tissue processing

Immediately after decapitation, the pancreas, spleen, liver and ovaries were dissected and frozen on dry ice, then transferred to an ultralow freezer (−80 °C) until peptide determinations were performed. A fragment of each structure was weighted. The tissues were then immersed in 2N acetic acid. The tubes were then heated in a boiling water bath for 10 min to inactivate proteolytic enzymes. The tissues were homogenized by sonication, the suspensions centrifuged for 10 min, and the supernatants aspirated, lyophilized, and kept at −20 °C until assayed. Before the determinations, the dry extract were suspended in 0.5 ml of the RIA buffer (0.5% BSA–PBS with aprotinin) and centrifuged to eliminate insoluble particles and dispensed into the assay tubes. 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) was used with good results.

2.4. Peptide quantification

Following the methodology previously used in the determinations of the values for NKA and SP in the hypothalamus and anterior pituitaries [6,8,9], we determined the concentrations of these TKs in extracts of ovaries, liver, spleen and pancreas. The specificity of the assay was confirmed by the presence of a single immunoreactive peak (corresponding to SP) in extracts of hypothalami [9]. Hypothalamic extracts of Siberian hamsters were purified by HPLC among synthetic SP and a single peak corresponding to SP was detected. The extraction of TKs from above-mentioned organs was performed following the same technique and reagents used for hypothalami and anterior pituitaries purified by HPLC.

For NKA we produced an antiserum by immunizing rabbits with NKA coupled to bovine thyrogobulin that showed low cross-reactivity with some TKs (0.78% with SP, 1.56% with neurokinin B, but high cross-reactivity with neuropeptide K (NPK) and neuropeptide gamma (NPγ). It must be pointed out that NPK and NPγ contain the whole sequence of NKA and therefore cross-react with this antiserum. This antiserum should be therefore considered to be able to bind NKA plus NKA contained in NPK and NPγ. This was confirmed by performing NKA assays in extracts of hypothalami [45] and anterior pituitaries purified by HPLC [8].

Synthetic SP and NKA (Cambridge Research Biochemicals, Wilmington, DE) were used as standards preparations. Standard curves with synthetic NKA and SP were set up with doses ranging from 2.5 to 1250 pg per tube. The

antisera were diluted in 1% normal rabbit serum in EDTA–PBS and dispensed in 200 ml per tube. Bolton–Hunter labeled ^{125}I-SP or ^{125}I-NKA (Amersham Corp., Arlington Heights, IL) were used as tracers. The labeled peptides were added to each tube in a volume of 100 μl containing 10,000 cpm. The incubation was carried out for 4 days at 4 °C and the separation between bound and free NKA or SP was achieved with the addition of a second antibody.

The results were expressed as pg of synthetic NKA or SP/mg tissue (Cambridge Research Biomedicals, Wilmington, DE).

2.5. Statistical analysis

Statistical analysis was performed using the SIGMA Statisticals Program (Copyright Horus Hardware, 1986). Results were expressed as mean ± S.E.M. Comparisons among ages were determined by ANOVA. Statistical differences were noted by (a) $P < 0.01$ or (b) $P < 0.05$. Differences between control and melatonin-treated females in each age were determined by *t*-test. Differences were noted by: $^{*}P < 0.01$, $^{**}P < 0.05$.

3. Results

3.1. Ovaries

NKA concentrations in control and melatonin-treated groups did not show significant differences for the three ages studied. Only significant lower values ($P < 0.01$) in the old–MEL as compared to old–control group were found. SP concentrations in the old–control group showed significantly increased ($P < 0.01$) values as compared to young– and middle-aged–control groups. Melatonin treatment resulted in lower SP concentrations in the old–MEL female rats than in old–control rats and consequently differences among the three ages studied disappeared. Neither differences between control and melatonin-treated groups were found (Fig. 1).

3.2. Pancreas

In the control group NKA concentrations were increasing from young to middle-aged up to old female rats, showing the young–control significantly lower values ($P < 0.01$) than middle-aged– and old–control groups. In the melatonin-treated group differences among ages studied disappeared, instead in the old–MEL group significantly lower values ($P < 0.05$) as compared to the old–control were found. SP concentrations in the control group increased from young– to middle-aged– up to old–control group, showing the old–control group significantly higher values ($P < 0.01$) as compared to the young–control group. In the melatonin-treated group, SP concentrations were significantly lower ($P < 0.05$) in old–MEL group as compared to old–control group. And melatonin treatment also decreased SP concentrations in middle-aged female rats showing significant differences ($P < 0.05$) as compared to old–MEL-treated rats (Fig. 2).

3.3. Liver

NKA and SP concentrations in the control and melatonin-treated groups did not show significant differences for the three ages studied. Similarly, not significant differences for NKA and SP in the three ages studied between control and melatonin-treated groups were found (Fig. 3).

3.4. Spleen

NKA concentrations in control and melatonin-treated groups increased from young to middle-aged up to old

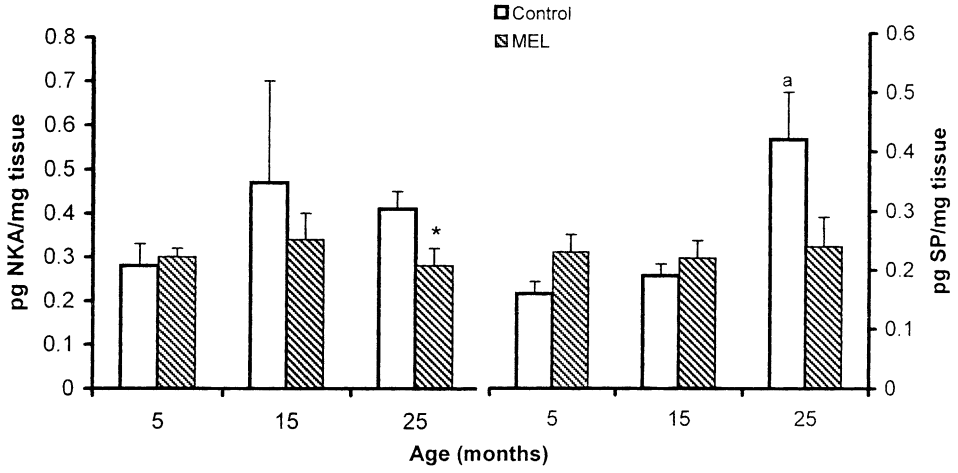

Fig. 1. Developmental pattern of NKA and SP immunoreactive substances in the ovary of control and melatonin-treated (150 μg/100 g BW) female rats. Data are presented as the mean ± S.E.M. at each age. The samples size were for NKA: $n = 7, 7, 7$ for the respective control age groups, $n = 7, 8, 7$ for the respective melatonin age groups. For SP: $n = 11, 11, 8$ for the respective control age groups, $n = 11, 12, 10$ for the respective melatonin age groups. (∗) $P < 0.01$ vs. control group. SP: (a) $P < 0.01$ vs. young– and middle-aged–control groups.

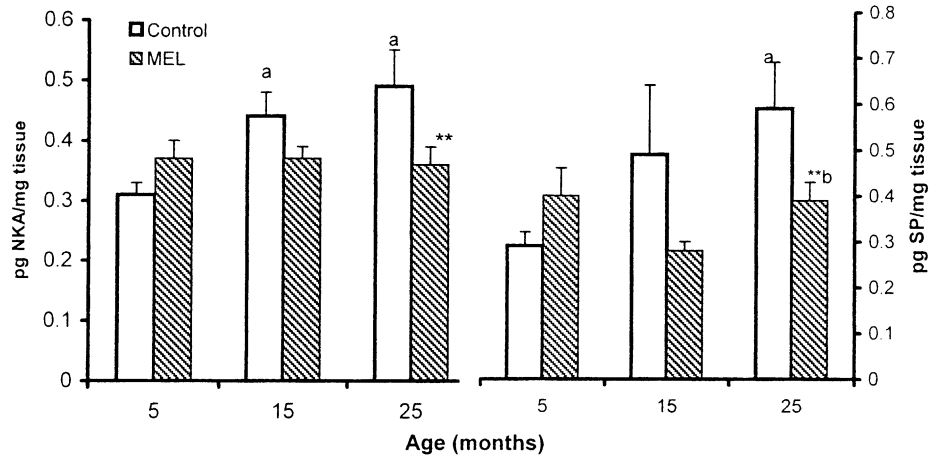

Fig. 2. Developmental pattern of NKA and SP immunoreactive substances in the pancreas. Data are presented as the mean ± S.E.M. at each age. The samples size were for NKA: $n = 15, 12, 12$ for the respective control age groups, $n = 20, 13, 15$ for the respective melatonin age groups. For SP: $n = 15, 11, 12$ for the respective control age groups, $n = 20, 12, 16$ for the respective melatonin age groups. (**) $P < 0.05$ vs. control group. NKA: (a) $P < 0.01$ vs. young–control. SP: (a) $P < 0.01$ vs. young–control; (b) $P < 0.05$ vs. middle-aged–MEL.

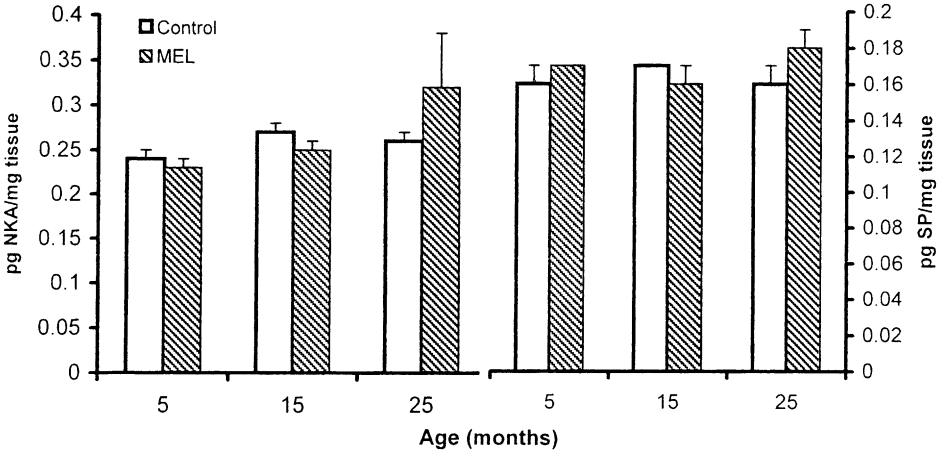

Fig. 3. Developmental pattern of NKA and SP immunoreactive substances in the liver. Data are presented as the mean ± S.E.M. at each age. The samples size were for NKA: $n = 26, 14, 15$ for the respective control age groups, $n = 23, 13, 17$ for the respective melatonin age groups. For SP: $n = 7, 8, 10$ for the respective control age groups, $n = 9, 8, 10$ for the respective melatonin age groups.

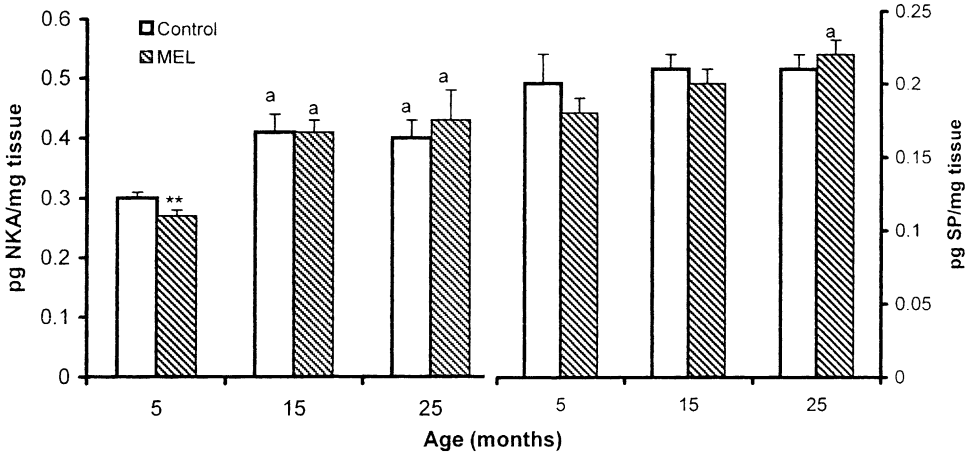

Fig. 4. Developmental pattern of NKA and SP immunoreactive substances in the spleen. Data are presented as the mean ± S.E.M. at each age. The samples size were for NKA: $n = 27, 15, 14$ for the respective control age groups, $n = 22, 14, 16$ for the respective melatonin age groups. For SP: $n = 16, 14, 14$ for the respective control age groups, $n = 19, 13, 16$ for the respective melatonin age groups. (**) $P < 0.01$ vs. control group. NKA: (a) $P < 0.01$ vs. young–control and vs. young–MEL. SP: (a) $P < 0.01$ vs. young–MEL.

female rats, having the young groups significantly lower values ($P < 0.01$) than the middle-aged and old groups. Melatonin treatment to young female rats produced significantly reduced values ($P < 0.05$) as compared to young–control group. SP concentrations in control group showed similar values at all ages studied, and no differences as compared to melatonin-treated groups were found. For the melatonin-treated groups in the old female rats significantly higher values ($P < 0.01$) than in young or middle-aged groups were observed (Fig. 4).

4. Discussion

The present results reveal that low concentrations of NKA and SP are present in the rat ovary regardless of their age. No significant differences in ovarian NKA concentrations in the three age groups studied were found. There are few reports on the role of TKs in the ovary, but some indicate that these peptides are present in the ovary and they may affect the secretion of ovarian steroids [7]. SP is involved in the neuroendocrine role of hypothalamo–pituitary–gonadal axis [9,10,28]. Differences in plasma concentrations of SP and NKA throughout the rat estrous cycle have been reported. Plasma concentrations of SP were increased in proestrus at 19:00 h, while NKA decreased, and higher concentrations of both peptides were observed on the day of proestrus in anterior pituitary as compared with the values in the other 3 other days of the cycle [14]. Our results show the highest ovarian SP concentration in 25-month-old rats, in which sexual cycles have disappeared. Ovarian steroids have been shown to affect SP and NKA concentrations in ovariectomized rats while injections of estradiol–benzoate (EB) decrease plasma concentrations of SP at the time when a peak of serum LH was detected; no effect of EB on the plasma concentration of NKA has been observed [15]. Previous data from our group [13] showed significantly increased plasma gonadotropins in 25-month-old female rats as compared to 5- or 23-month-old rats, and no differences in estradiol or progesterone levels among the three ages studied were found; in this study the young rats were studied during the diestrous phase of the sexual cycle. Consequently from our data was concluded that TK levels are regulated not only by ovarian steroids but also by gonadotropins. Also, in our laboratory, significantly increased hypothalamic NKA and SP concentrations were found in the same 25-month-old female rats [18]. Both hypothalamic TKs and plasma gonadotropins were significantly reduced by melatonin treatment. Similarly melatonin treatment significantly reduced the ovarian TK concentrations in the old rats, to the level observed in the younger animals.

Peptides are particularly important in the coordination of pancreatic exocrine and endocrine secretions and are similarly distributed in both the normal and diabetic rat pancreas [1]. Results obtained in chronic pancreatis suggest that SP is synthesized outside the pancreas [11]. It was previously reported that SP induced a marked, significant increase in insulin and glucagon secretion from the normal rat pancreas, but inhibited the release of these hormones from the diabetic rat pancreas [2,36]. Also, SP inhibited CCK-induced amylase release and secretion induced secretory flow in a dose-related manner [24]. Our results show significantly higher SP and NKA concentrations in the pancreas of old female rats as compared with the two other ages studied. This increase was blunted by melatonin treatment being lowered to values similar to those observed in young rats. This indicates a positive and beneficial effect of melatonin on pancreatic TK concentrations. No data are available on the influence of melatonin on pancreatic TK concentrations. The influence of melatonin on carbohydrate metabolism through the action of melatonin on pancreatic β-cells has been reported. These cells are known to contain melatonin-binding receptors [33]. Previous data have indicated that the removal of the endogenous source of melatonin in rodents, i.e. by pinealectomy, induced hypoglycemia and hyperinsulinemia [5,26], and reduced glucose-induced insulin secretion in both mice and rats [3,12,25]. More recently, a direct action of melatonin on the pancreatic β-cells activity which regulate both glucose metabolism and insulin secretion, has been described [19].

The rat hepatobiliary tract is prominently innervated by CGRP fibers which also contain SP-TK immunoreactive axons [20]. Also, studies in mice [29] found that SP might be selectively stimulated in the liver by bacterial infections. In our study, no modifications in liver TK concentrations in young and older female rats were observed. Melatonin had no influence on these concentrations. However, a protective effect of melatonin against oxidative damage to nuclear DNA and microsomal and mitochondrial membranes caused by delta-aminolevulinic acid in the rat liver has been reported [23]. Melatonin treatment reduced the damage associated with zymosan administration in the liver and other tissues [17]. Zymosan is a non-bacterial agent that causes inflammation.

TKs released from sensory neurons, act not only as neurotransmitters but also as immunological mediators [16,42]. TK-deficient (PPT-A (-1-)) mice infected with murine gamma herpes virus had delayed clearance of latently infected cells from the spleen [32]. Our results suggest a relevant role for NKA during aging, since higher spleen NKA concentrations in middle-aged and old control and melatonin-treated female rats were found. SP, often considered a regulatory peptide that may play a role in immune regulation, has been detected in the central arteries of spleen [39]. However, it did not show significant changes through aging in the control rats but in melatonin-treated animals increased values in old rats were observed.

The current results are relevant to the understanding of the interaction between TKs in various organs, and to the possible influence of melatonin on the changes during aging.

Acknowledgments

This work has been supported for the Spanish Ministry of Health, Fondo de Investigación Sanitaria. FIS No. 97/0988. The authors wish to express their gratitude to Dr. Reiter, University of Texas, Health Science Center at San Antonio, San Antonio, TX, USA, for his help in the English edition.

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