Developmental changes of hypothalamic, pituitary and striatal tachykinins in response to testosterone: influence of prenatal melatonin

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Abstract

Substance P (SP) and neurokinin A (NKA), members of the family of mammalian tachykinins, are involved in the regulation of many physiological functions and are widely distributed in mammalian tissues. In this report, the effects of prenatal melatonin on the postnatal developmental pattern of NKA, and SP, and on testosterone secretion were investigated. Also, tachykinin response to the administration of testosterone propionate (TP) was studied. The brain areas studied were medio-basal-hypothalamus, pituitary gland and striatum. Male rat offspring of control or melatonin treated mother rats were studied at different ages of the sexual development: infantile, juvenile or prepubertal periods, and pubertal period. Both groups received exogenous TP (control-offspring + TP and MEL-offspring + TP), or the vehicle (control-offspring + placebo and MEL-offspring + placebo). Hypothalamic concentrations of all peptides studied in control-offspring + placebo remained at low levels until the juvenile period, days 30–31 of age. After this age, increasing concentrations of these peptides were found, with peak values at puberty, 40–41 days of age, then declining until adulthood. In the MEL-offspring + placebo a different pattern of development was observed; hypothalamic concentrations of NKA and SP from the infantile period until the end of juvenile period were significantly higher than in control-offspring + placebo. TP administration exerted a more marked influence on MEL-offspring than on control-offspring and prevented the elevation in tachykinin concentrations associated with prenatal melatonin treatment. TP administration to control-offspring resulted in significantly reduced (P < 0.05) tachykinin concentration only at 40–41 days of age, and increased (P < 0.01) during infantile period as compared to control-offspring + placebo. Pituitary NKA concentrations were lower than in the hypothalamus. In control-offspring + placebo pituitary NKA levels did not show significant changes throughout sexual development. A different developmental pattern was observed in MEL-offspring + placebo, with significantly increased (P < 0.05) pituitary NKA concentrations at 35–36 days of age than in control-offspring + placebo. TP administration to control-offspring influenced pituitary NKA levels at the end of the infantile and pubertal periods, showing at both stages significantly higher (P < 0.05) NKA levels as compared to control-offspring + placebo. NKA levels in MEL-offspring + TP were only affected at 21–22 days of age, showing significantly increased (P < 0.01) values as compared to MEL-offspring + placebo. Striatal tachykinin concentrations in control-offspring did not undergo important modifications throughout sexual development, but during the prepubertal period they started to increase. Maternal melatonin and TP injections produced short-lived alterations during the infantile period. The results showed that prenatal melatonin delayed the postnatal testosterone secretion pattern until the end of the pubertal period and postnatal peptide secretion in brain structures. Consequently, all functions depending of the affected areas will in turn, be affected. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: NKA; SP; Prenatal melatonin; Hypothalamus; Pituitary; Striatum; Testosterone

1. Introduction

Substance P (SP) and neurokinin A (NKA) are members of the family of mammalian tachykinins [20,29] and are widely distributed in mammalian tissues. NKA is a decapeptide contained in the same neurons that also contain SP [29], and many of its effects are similar to those of SP on neurons and peripheral tissues.

Previous studies demonstrated that the preprotachykinin gene is expressed in the rat anterior pituitary [21], suggesting that this tissue is a site of tachykinin synthesis. SP and NKA were found in the rat anterior pituitary [6,34], and in hypothalamic areas related to the control of reproduction, and of sexual and feeding behavior [4,5,22]. The effect of
gonadal steroids and photoperiod were studied on SP containing neurons in areas involved in sexual behavior. Hypothalamic SP decreased after castration or under decreased day length, and testosterone treatment prevented this decrease [31]. Previous results indicated that testosterone regulates the SP and NKA contents in the hypothalamus of male Syrian golden hamsters [27,32], in the hypothalamus and the anterior pituitary gland of rats and mice [2,5]. Melatonin can be transported from the blood into compartments such as amniotic fluid [26], and can cross the placental barrier in rats [23] as well as in non-human primates [30], and can modify the function of many tissues and organs. The regulation of physiological functions by the pineal gland and melatonin of different brain areas during prenatal and postnatal periods still remains unknown. Previous findings suggest a direct influence of maternal melatonin on the postnatal development of rats [10,19], voles (Microtus montanus) [15,16] and Siberian hamsters [12]. Because the pineal gland has receptors for SP [13] we considered important to study if maternal melatonin could affect the postnatal development of NKA, and SP stores in hypothalamus, pituitary gland and striatum in male rats. This could clarify 2 important points; 1) a possible melatonin effect on neuropeptide biosynthesis during intrauterine life, and 2) duration or time of melatonin effect throughout postnatal development.

There is now evidence that striatum activity is modulated by melatonin [3]. Also, a positive influence of 5-HT on SP biosynthesis, similar to the effects of dopamine, has been observed [1]. Recent results from our laboratory have also demonstrated an important influence of maternal melatonin on striatal tachykinins in female rats. It is known that serotonin N-acetyltransferase (arylalkylamine N-acetyltransferase, AANAT) is the enzyme that controls the daily rhythm in pineal melatonin production [25]. Recently the striatum has been included in the selected brain areas that AANAT gene has been detected in significant levels [24]. Considering these functional characteristics we speculated that the inclusion of the striatum in the present investigation could add further knowledge to the role of maternal melatonin in the postnatal development.

In the present investigation, the effects of maternal melatonin on postnatal development of concentrations of NKA and SP in basal hypothalamus, anterior pituitary gland and striatum were studied under physiological and supraphysiological dose ranges of gonadal steroids, from the infantile period until adulthood. We considered that those studies could be of considerable interest because a developmental pattern of tachykinins in male offspring has not been reported.

2. Materials and methods

2.1. Animals and treatments

Female Wistar rats from our colony and weighing 240–280 g at the beginning of the experiment were used. Animals were housed under 12-h light/dark cycles (lights on at 8:00 a.m.), at a room temperature of approximately 23°C. Standard rat chow and water were available ad lib. Mother rats were divided into 2 groups: control (N = 48) and melatonin-treated (N = 30), mating pairs were kept in polypropylene cages, one male with 2 females. Possible pregnancy was monitored by the presence of vaginal spermatozoa. Considering previous findings [23] that 20 μCi of 3H-acetyl-melatonin was administered to pregnant rats, and that each fetus contained slightly more than 0.1% (20 nCi), of the injection dose, 150 μg MEL/100 g body weight were used in the present study. MEL (Sigma Chemical Co., St. Louis, MO) was dissolved in a small volume of absolute ethanol and diluted in 0.9% NaCl to a dose of 150 μg/100 g body weight. Melatonin treatment was given by SC injection at end of the light phase, and daily throughout pregnancy. Control mother rats received ethanol/saline alone.

At delivery, litter sex and number of pups were recorded. To obtain uniformity in the development of the pups, on the day of birth each litter was adjusted to 12 pups per dam by cross-fostering some pups from larger litters within treatment groups. Pups remained with the mother until weaning on Day 21 (birth = Day 0). To study male offspring we followed the classification by Øjeda [28] concerning postnatal maturation: a) Infantile period, between 8 and 21 days, animals were studied at 17 (Control n = 26; MEL n = 24), and 21 (Control n = 21; MEL n = 22) days of age; b) Juvenile or prepubertal period, from 21 to 35 days of age; animals were studied at 30 (Control n = 26; MEL n = 25) or 35 (Control n = 25; MEL n = 26) days of age; c) Pubertal period, from Day 35 to 55–60 days of age, animals being examined at 40 (Control n = 35; MEL n = 25), 60 (Control n = 22; MEL n = 18) days of age. At the mentioned ages the animals were killed by decapitation. Blood was collected from the trunk, and plasma was stored at −20°C until assay of testosterone was performed. The animals were divided into 2 groups: offspring of control mother rats (Control offspring) and offspring of MEL treated mother rats (MEL offspring).

2.1.1. Testosterone treatment

Offspring of male rats was treated with testosterone propionate (TP). (Control offspring received TP; Control offspring + TP group. MEL offspring received TP; MEL offspring + TP group). Offspring received placebo: (Control offspring+placebo group and MEL offspring+placebo group). TP (Sigma Chemical Co., St. Louis, MO) was dissolved in polyethylene glycol and injected at a single dose of 100 μg/100 g BW. A volume of the solution lower than 0.5 ml was administered by SC injection to each animal at the above mentioned ages, the animals that received TP were killed one day after the injection.
2.2. Tissue processing

Immediately after decapitation, the skull was quickly opened, brain and pituitaries were removed and frozen on dry ice, then transferred to an ultra-low freezer (−80°C) until peptide determinations were performed. The hypothalamus was cut in a single fragment of approximately 3–5 mg, following the anterior limits given by the optic chiasma and the posterior by the mamillary bodies, and the lateral cuts along the lateral sulci. The striata, and pituitary gland were dissected out. Tissues from each animal were immersed in 0.5 ml of ice-cold 2N acetic acid contained in Eppendorf tubes. The tissues were then heated in a boiling water bath for 10 min, to inactivate proteolytic enzymes, homogenized by sonication, centrifuged for 10 min, and the supernatant was aspirated, lyophilized, and kept at −20°C until assayed. Protein concentrations in each tissue extract were determined by the Bradford method using bovine serum albumin (BSA) as standard.

2.3. Peptide quantification

The concentrations of NKA and SP were determined in basal hypothalamus, pituitary gland and striatum. In pituitary gland only NKA was determined. SP and NKA were determined by double antibody radioimmunoassays previously described [4,8,9]. SP was determined using an anti-SP serum obtained in rabbits. The specificity of the assay was confirmed by the presence of a single immunoreactive peak (corresponding to SP) in extracts of hypothalami, anterior pituitaries and striatum purified by HPLC [7].

NKA was determined in each hypothalamic, pituitary, and striatum extracts by a radioimmunoassay method using an antiserum that we produced by immunizing rabbits with NKA coupled to bovine thyroglobulin. It must be pointed out that neuropeptide K (NPK) and neuropeptide gamma (NPγ) contain the whole sequence of NKA and therefore crossreact 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 and anterior pituitaries, purified by HPLC [7].

Before being assayed, each hypothalamic extract was redissolved in 0.4 ml of 0.1 N acetic acid. Five to 10 μl of this extracts were aliquoted into test tubes in duplicates, the volume was completed up to 0.5 ml with 0.5% BSA in phosphate-saline buffer. Before performing the assays, the anterior pituitary extracts were dissolved in 500 μl of assay buffer (0.5% BSA, Sigma Chemical Co, St Louis, MO; bacitracin 20 μM, PBS). The choice of BSA may be critical, because some albumins apparently contain proteolytic activity, that may render the assay unsuccessful. In these assays BSA isolated by heat shock (Sigma Chemical Co., St Louis, MO) was used with good results. The solution was centrifuged to eliminate insoluble particles, and 500 μg (equivalent to 1 anterior pituitary) was dispensed into each assay tube.

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/tube. The antisera were diluted in 1% normal rabbit serum in EDTA-PBS and dispensed in 200 μl/tube. Bolton-Hunter labeled 125I-SP or 125I-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 (Cambridge Research Biochemicals, Wilmington, DE)/μg protein.

2.4. Radioimmunoassay of testosterone

Plasma testosterone was measured by 125I RIA Kit using commercial ImmunoChem® Coated Tube, according to the manufacturer’s instructions (ICN Biomedicals Inc, Costa Mesa, CA 92626). The minimum amount of testosterone significantly different from zero was 0.2 ng/ml. All samples were measured with kits belonging to the same catalogo’s reference. The assay binding was 52.86%.

2.5. Statistical analysis

Data of each age group were adjusted to a normal distribution test before being used in the statistical analysis. A 99% of accuracy to normal distribution was required. Statistical analysis was performed using the SIGMA Statistical program (Copyright Horus Hardware, 1986). Results were expressed as mean ± SEM. Comparisons among groups of data of tachykinin concentrations or hormone blood levels at each time point studied were determined by one-way ANOVA; individual comparisons across ages within each treatment groups were then made by Newman–Keuls multi-range test, Mann–Whitney test was used for those cases with borderline significant values from ANOVA. Probability values lower than P < 0.05 were considered significant.

Only the following comparisons were considered: Control-offspring+TP versus MEL-offspring+TP and Control-offspring+placebo versus MEL-offspring +placebo (*P < 0.01 or **P < 0.05); Control-offspring+TP versus Control-offspring+placebo and MEL-offspring+TP versus MEL-offspring+placebo (a: P < 0.01 or b: P < 0.05).

3. Results

3.1. Hypothalamus

In control-offspring+placebo low values of hypothalamic NKA concentrations during infantile period were
found, that were significantly lower ($P < 0.01$) than in control-offspring+TP (Fig. 1). During the prepubertal period values started to increase and during the pubertal period NKA concentrations in control-offspring+placebo were significantly higher ($P < 0.05$) than in control-offspring+TP, and, at the end of pubertal period, the values again decreased, being similar to those found in control-offspring+TP. In MEL-offspring+placebo hypothalamic NKA concentrations started to increase earlier than in control offspring and these concentrations were the highest observed in all groups studied, having significantly higher values ($P < 0.01$) at 17–18, 30–31, 35–36 and 60–61 days of age than in control-offspring+placebo and also at 40–41 days of age ($P < 0.01$) than in MEL-offspring+TP. At 17–18 days of age, significantly higher ($P < 0.01$) values in control-offspring+TP than in MEL-offspring+TP were found. After this age the values were higher at 30–31 days of age but without reaching significant differences.

In control-offspring+placebo a SP developmental pattern similar to that observed in NKA was found: low values during infantile period and until the beginning of prepubertal phase were found, then they started to increase reaching higher values at 40–41 days of age and showing significantly higher values ($P < 0.05$) than control-offspring+TP, again at 60–61 days of age, the values decreased (Fig. 2). In control-offspring+TP hypothalamic SP concentrations showed values around 9 pg/µg protein at all ages studied. In MEL-offspring+TP SP concentrations in the hypothalamus were close to those observed in control-offspring+TP except at 17–18 days of age when in MEL-offspring+TP significantly lower values ($P < 0.05$) than control-offspring+TP were found. In MEL-offspring+placebo coinciding with data observed for NKA, SP concentrations showed the highest values observed with peak values at 35–36 days of age having significantly higher values ($P < 0.01$) from infantile until the beginning of pubertal period than in MEL-offspring+TP. During the infantile and prepubertal periods and until the beginning of pubertal period 40–41 days of age, also MEL-offspring+placebo group showed significantly higher values ($P < 0.01$) as compared to control-offspring+placebo group.
3.2. Pituitary gland

NKA concentrations in the anterior pituitary were lower than in hypothalamus (Fig. 3). In control-offspring + placebo the developmental pattern of NKA concentrations remained at similar level independently of the sexual stage. TP administration in control offspring only showed significantly higher concentrations (P < 0.05) at 21–22 and 60–61 days of age as compared to control-offspring + placebo. However in MEL-offspring again the pattern of development was different. A decrease of NKA concentrations was delayed until days 21–22 of age. After-pubertal period similarly to what was found in basal hypothalamus showing in MEL-offspring + placebo significantly higher NKA concentrations (P < 0.05) at 35–36 days of age than in control-offspring + placebo. A similar response was observed in MEL-offspring + TP, NKA concentrations were significantly higher (P < 0.05) than in control-offspring + TP at 40–41 days of age. Also at 21–22 days of age significantly higher (P < 0.01) NKA concentrations in MEL-offspring + TP than in MEL-offspring + placebo were found.

3.3. Striatum

Concentrations of NKA in striatum were much lower than in the hypothalamus (Fig. 4). In control-offspring + placebo extremely low values were found during infantile period. These were significantly lower (P < 0.01) than in control-offspring + TP at 21–22 days of age. During prepubertal period values increased reaching levels similar to those observed in control-offspring + TP. In MEL-offspring + placebo at 17–18 days of age significantly higher NKA concentrations (P < 0.01) than in control-offspring + placebo were found, after this age values decreased to levels similar to other groups studied. In MEL-offspring + TP striatal NKA concentrations ranged 1 pg/μg protein during all developmental phases studied, showing significantly higher values (P < 0.05) at 21–22 and 40–41 days of age than in MEL-offspring + placebo.

Striatal SP concentrations in the same groups studied were much lower than in hypothalamus (Fig. 5). As it was observed with NKA, in control-offspring + placebo, SP concentrations were extremely low during the infantile period showing significantly lower values (P < 0.01) than in control-offspring + TP at 21–22 days of age, then values increased having significantly higher values (P < 0.05) than in MEL-offspring + placebo at 40–41 days of age. In MEL-offspring + placebo striatal SP concentrations showed sig-
significantly higher values ($P < 0.01$) than in control offspring + placebo and than in MEL-offspring + TP at 17–18 days of age, then the concentrations decreased abruptly. In MEL-offspring + TP values remained at low levels throughout all ages studied.

In control-offspring + TP and MEL-offspring + TP, testosterone levels were detectable through all phases of sexual development (Fig. 6). In control-offspring + placebo testosterone values were found at 17–18 days of age but then not detectable values were observed until the end of prepubertal period, and testosterone concentrations increased through the pubertal period. In MEL-offspring + placebo detectable testosterone values were found only at 17–18 days of age and then delayed until 60–61 days of age, being values significantly higher ($P < 0.05$) than in MEL-offspring + TP.

4. Discussion

In recent years there has been an increasing interest in the effects of melatonin on the control of brain function. However, the sites and mechanism of melatonin action are not completely known. The results of this investigation show that exposure to prenatal melatonin resulted in profound alterations of the hypothalamic and pituitary tachykinin developmental pattern. Treatment with PT during development prevented the elevation of tachykinins levels associ-
ated with prenatal melatonin treatment. It is known that the effects of bilateral enucleation as well as melatonin treatment may resemble those of short photoperiod [14,17]. Bilateral enucleation of golden hamsters produced a decrease in the number of SP neurons in areas of limbic system involved in the mediation of steroid actions on male reproductive behavior, and testosterone treatment prevented this decrease [31]. In the MEL-offspring + placebo group, however, an opposite response, with increased NKA and SP concentrations was observed. In control-offspring, administration of TP during infantile period resulted in increased hypothalamic NKA levels. When the animals started to secrete their own steroids at 35–40 days of age, testosterone treatment inhibited the peak values. In relationship to this, in young male rats it was found that either prolonged or acute castration was followed by a decrease of NKA concentrations in the hypothalamus of male rats. The replacement treatment with TP for 14 days resulted in an increase of hypothalamic NKA above the values found in intact animals [6]. All these data indicate that tachykinin concentrations in basal hypothalamus may be regulated by testosterone. And our results show for the first time that prenatal melatonin delayed the onset of testosterone secretion until the end of pubertal age in male offspring and regulated the postnatal developmental pattern of tachykinins in the hypothalamus.

The concentrations of NKA in the pituitary gland were tenfold lower than in the hypothalamus, similar results were previously reported in the Syrian (golden) hamster or the rat [4]. It has been reported that the biosynthesis of NKA in anterior pituitary gland is influenced by gonadal steroids [21]. Pituitary NKA concentrations showed decreased values in castrated male rats that were restored at levels similar to those found in intact rats by testosterone and dihydrotestosterone replacement treatment [5]. Our results also suggest that the ability of exogenous TP to modulate tachykinin concentration is less marked in the pituitary gland than in the hypothalamus during sexual development. Only during the infantile period, at 21 days of age when no detectable testosterone levels were observed, TP treatment produced significantly increased NKA concentrations in both control- and MEL-offspring. During the prepubertal period, when all sexual mechanisms are maturing, anterior pituitaries were no responsive to exogenous TP. At the end of pubertal period, however, the response was again observed in control-offspring.

Striatal NKA and SP concentrations of control rats, did not undergo marked modifications throughout sexual development. However, during the juvenile period, tachykinin concentrations started to increase that confirms previous findings of SP receptor mediating a complex combination of presynaptic and postsynaptic functions in the mammalian striatum [18]. Melatonin treatment of the mother produced acute but short-lived alterations on postnatal striatal tachykinins pattern. Our results are supported by previous works that administration of melatonin [3] influenced the striatal neurons activity, or the melatonin precursor serotonin [1] the striatal SP biosynthesis.

Whether all these changes in the developmental patterns of tachykinins are caused by a direct action of melatonin on hypothalamus, pituitary gland or striatum, or alternatively, indirectly through melatonin influence on other structures, remains to be clarified. But the surprising finding of strong expression of the AANAT gene in the pituitary gland, hypothalamus and striatum [24], suggest that a direct action of maternal melatonin on these structures is possible. Furthermore, the suprachiasmatic nucleus of the hypothalamus is among the brain areas that are most frequently mentioned as a potential site of melatonin action in the rat [33]. In the rat [35], and the Syrian hamster [36] melatonin binding sites were localized in the pars tuberalis of the anterior pituitary and hamster melatonin binding sites were localized in the striatum [11].

The present data provide new information on melatonin effects on the CNS, the main target tissue for the pineal hormone. Our data suggest that melatonin is able to modulate concentrations of some neurotransmitters during the intrauterine life as well as the developmental secretion pattern of testosterone in offspring. As brain functions are regulated by neurotransmitters, consequently, all functions depending of the affected areas will, in turn, be affected.

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