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Effects of pinealectomy and melatonin on gonadotropin-releasing hormone (GnRH) gene expression in the male rat brain

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Melatonin, a pineal hormone, is known to be an important neurohormonal factor involved in the timing of reproductive events which occur seasonally in various mammalian species. In order to evaluate the influence of melatonin on neurons which are producing gonadotropin-releasing hormone (GnRH), we studied the effect of light-dark cycle as well as pinealectomy and melatonin administration on GnRH gene expression in the adult male rat medial preoptic area (MPOA) using quantitative *in situ* hybridization. The animals were kept under artificial light (light on 6:00 h–20:00 h). In animals which were sacrificed at 24:00 h (when endogenous melatonin levels are high), the hybridization signal was higher than that detected in animals sacrificed at 20:00 h (before the onset of darkness). Administration of melatonin during the light period (16:00 h) induced a 15% increase in the amount of GnRH mRNA after 4 h. Three weeks after pinealectomy mRNA levels were decreased by 35%. Injection of melatonin to pinealectomized rats 4 h before sacrifice increase the amount of GnRH mRNA, completely reversing the decrease in mRNA induced by pinealectomy. These results strongly suggest that melatonin produced by the pineal gland exerts a positive influence on GnRH neuronal activity in the male rat.

Keywords: Melatonin; pineal gland; gonadotropin-releasing hormone; light-dark cycle

Introduction

Melatonin, which is a major product of the pineal gland, is considered as an important neurohormonal intermediate involved in the timing of reproductive events in various mammalian species (Lincoln, 1984; Tamarkin *et al.*, 1985). The effects of melatonin on reproduction have also been shown to vary as a function of the experimental conditions. In immature male rats, chronic injection of melatonin from 20 to 40 days of age reduced weights of testis and seminal vesicles, indicating that melatonin delayed sexual maturation at 40 days (Lang *et al.*, 1984). In the female animal, the daily administration of melatonin from the day 15 of age resulted in a 10-day delay of puberty as evaluated by vaginal opening (Rivest *et al.*, 1985). In the adult male rat, melatonin treatment decreased testis weight, spermatogenesis and plasma testosterone levels (Kinson & Peat, 1971; Konig & Rega, 1978; Mas *et al.*, 1979). On the other hand, in the adult male rat, it has been reported that melatonin could reverse the antigonadotrophic effects of blinding (Chen & Reiter, 1980). In pubertal hamsters, pinealectomy markedly reduced the testicular development (Kelley *et al.*, 1994). The influence of melatonin on reproductive neuroendocrine axis suggests that, in the rat as in many other species, melatonin secretion which is regulated by the light-dark cycle may be involved in the regulation of gonadotropin secretion (Lincoln, 1984; Tamarkin *et al.*, 1985). Melatonin may act the level of the central nervous system and/or directly on the pituitary gland to modify gonadotropin secretion. So far, the precise site(s) of action of melatonin has not been clearly elucidated, although the distribution of melatonin receptors suggests that the pars tuberalis of the pituitary as well as the medial preoptic area (MPOA) (Weaver *et al.*, 1985; Williams *et al.*, 1989, 1991) which contains a large number of gonadotropin-releasing hormone (GnRH) neurons (Pelletier, 1980; Silverman *et al.*, 1987) might be possibly the structures involved in the effects of melatonin on gonadotropin secretion.

In an effort to examine in more details the effects of melatonin on GnRH-producing neurons, we studied the influence of light-dark cycle as well as pinealectomy and melatonin administration on GnRH gene expression in the male rat MPOA.

Results

Analysis of the autoradiograms indicated that a strong signal could be obtained after 20 days of exposure (Figure 2). The specificity of the signal could be established by the following findings: (1) labelled cells have the same localization as that reported by immunocytochemical staining of GnRH (Pelletier, 1980; Silverman *et al.*, 1987); (2) hybridization with a sense oligomer produced no labelling in adjacent sections; (3) pretreatment with RNase completely prevented any labelling following hybridization with the labelled antisense probe. Moreover, using a combination of immunocytochemistry and *in situ* hybridization, we have previously shown that hybridization signal could be detected only in neurons containing immunoreactive GnRH (Toranzo *et al.*, 1989).

Cells expressing GnRH mRNA were observed by decreasing order of intensity in the preoptic area, anterior hypothalamus, diagonal band of Broca, and ventral septal area. The greater number of labelled cells (24 in a single 10 μm section) was observed in the area of the organum vasculosum of the lamina terminalis (OVLT).

As illustrated in Figure 1, in the animals which were sacrificed at midnight, the number of silver grains overyling labelled neurons appeared to be slightly but significantly higher than those evaluated in animals killed just before the onset of darkness (20:00 h). Administration of melatonin during the light period (16:00 h) induced 4 h later just before the onset of darkness a 15% increase in the hybridization signal (Figure 1).

Three weeks after pinealectomy, the mean value of silver grains per cell was decreased by 35% (Figures 2 and 3). Administration of melatonin to pinealectomized rats 4 h before sacrifice increased the amount of GnRH mRNA (27% over that measured in pinealectomized control rats), thus completely reversing the decrease in mRNA levels induced by pinealectomy (Figures 2 and 3).

Discussion

In the laboratory rat, which does not show an annual reproductive cycle, the production of melatonin by the pineal

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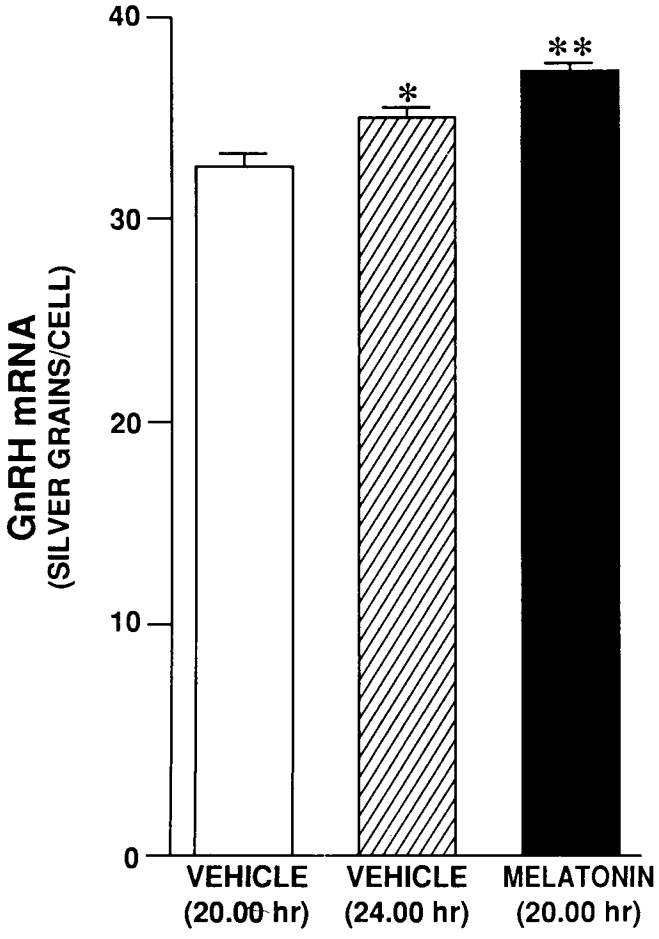

Figure 1 Effect of alternating light and dark period and melatonin administration on GnRH mRNA levels as measured by the number of silver grains overlying labelled neurons. The animals were killed either at the end of the light period (20:00 h) or 4 h after the onset of the dark period (24:00 h). Melatonin was injected 4 h before sacrifice. $*P<0.05$, $**P<0.01$, vehicle-treated (20:00 h) *vs* the other groups

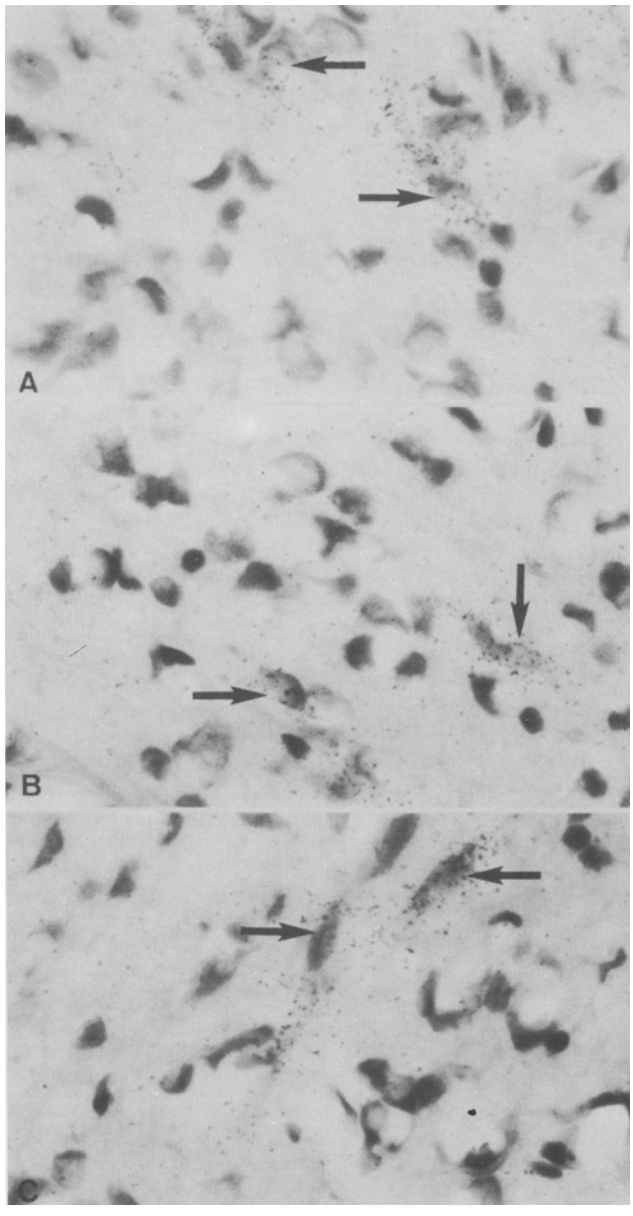

Figure 2 Light microscope autoradiographs illustrating the signal obtained in MPOA following hybridization with labelled GnRH probe. Silver grains are concentrated over a few neurons (→). X800. (**A**) Sham-oeprated; (**B**) pinealectomized; (**C**) pinealectomized rats treated with melatonin

gland vary rythmically during the light-dark cycle with a peak during the hours of darkness (Hoffmann, 1969; Klein & Weller, 1970; Lynch, 1971). In the present experiments, quantitative *in situ* hybridization was used to study the influence of light-dark cycles as well as the effects of pinealectomy and melatonin administration on the levels of GnRH mRNA in individual neurons in the male rat brain.

The results clearly indicate that GnRH mRNA levels are higher during the darkness period than during the light period and markedly decreased following long-term (3 weeks) pinealectomy. In both intact and pinealectomized animals, injection of melatonin injected during the light period induced 4 h later an increase in GnRH gene expression. It thus appears that melatonin can exert a positive influence on GnRH neuronal activity in the male rat.

These data are consistent with previous results indicating that, in the male hamster, pinealectomy prevent testicular development in juvenile animals (Kelley *et al.*, 1994). On the other hand, it has been consistently demonstrated that in the adult hamster gonadal regression and low serum gonadotropin levels can be induced by exposure to short days which are responsible for an increase in melatonin secretion (Simpson *et al.*, 1982; Yellon & Goldman, 1984, 1987). In the adult male rat, a reversal of antigonadotrophic effects of blinding by melatonin has been reported (Chen & Reiter, 1980) while, in the developing male rat (20–40 days of age), chronic administration of melatonin induced a delay in sexual maturation (Lang *et al.*, 1984). In the female, melatonin seems to exert a negative influence leading to delay in sexual maturation (Rivest *et al.*, 1985) and abolition of the preovulatory LH surge and ovulation (Ying & Greep, 1973; Trentini *et al.*, 1992). The discrepancies observed between the present results on GnRH gene expression and some previous reports on gonadotropin secretion might be tentatively explained by variations between species and sexes as well as by differences in experimental conditions. It remains possible that the decrease in gonadotropin secretion induced by melatonin might be mainly related to an inhibitory action of the indoleamine at the pituitary level and not to an influence on GnRH neuronal activity. In fact, it has already been shown that melatonin can directly suppress basal LH release from pituitary *in vitro* (Wun *et al.*, 1986a,b).

In the present study, the dose of melatonin which we used was higher than those required to counter the antigonadotrophic actions of the melatonin (Chen & Reiter, 1980). This might suggest that the stimulating effect of the indolamine on GnRH expression was pharmacological rather than

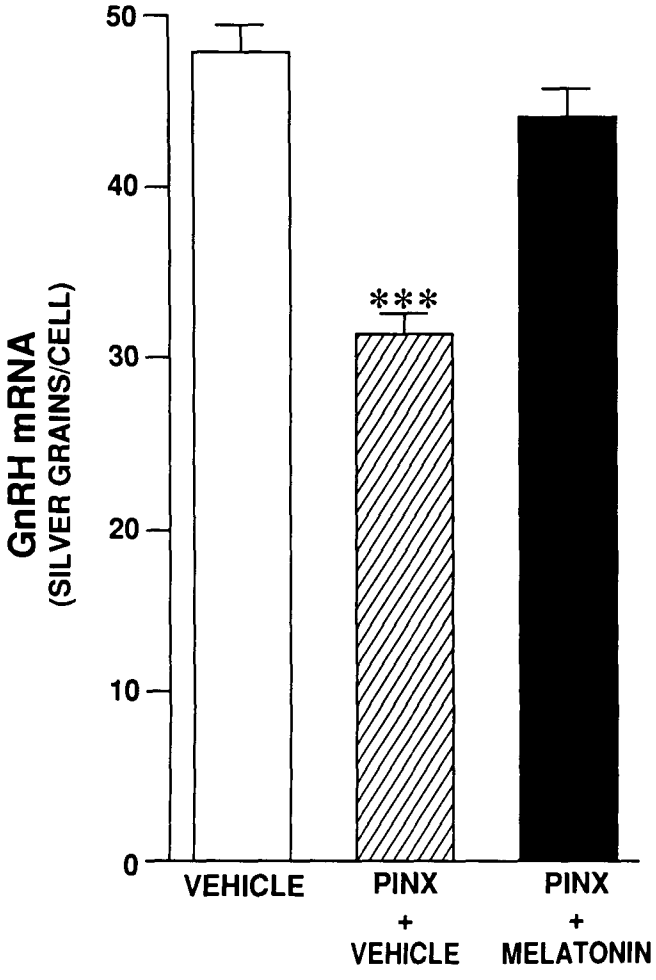

Figure 3 Effect of pinealectomy and melatonin administration to pinealectomized animals on GnRH mRNA levels. Melatonin was injected 4 h before sacrifice. ***$P<0.001$, vehicle-treated *vs* the other groups

physiological. On the other hand, since pinealectomy produced a marked decrease in GnRH mRNA levels, an effect which was reversed by melatonin administration, it means that the effect observed was probably directly related to an increase in circulating levels of melatonin.

Although the present data do not provide precise information about the exact site(s) of action of melatonin on GnRH neurons, it might be suggested that melatonin could exert a direct action on GnRH perikarya in the MPOA since melatonin receptors have been detected in this area (Williams *et al.*, 1989). Melatonin in general circulation can easily reach GnRH perikarya which are mostly located close to OVLT which lacks blood brain barriers (Pelletier, 1980; Silverman *et al.*, 1987). Moreover, it cannot be totally excluded that melatonin might affect the activity of neurotransmitter systems which normally regulate GnRH secretion. The local administration of melatonin in the different regions of the brain which contain specific high-affinity binding of melatonin is required to firmly establish the site(s) of action of the indolamine on the GnRH neuronal system.

In conclusion, the present results clearly demonstrate that melatonin produced by the pineal is involved in the positive regulation of GnRH gene expression in GnRH neurons in the adult male rats.

Materials and methods

Animals and experimental procedures

In a first series of experiments, 18 male rats weighing 225–250 g (purchased from Charles River Canada Inc.) were used. All the rats were housed under artificial light (light on 06:00–20:00) and temperature control (22 ± 1°C). After 2 weeks of adaptation to the light-dark condition, the animals were divided into three groups (6 rats/group). One group of rats received vehicle (0.5 ml) by intraperitoneal injection at 20:00 h and perfused at 24:00 h wih 4% paraformaldehyde (PF) for histological procedures. The two other groups received either vehicle (0.5 ml) or melatonin (24 mg/kg) at 16:00 h and were perfused just before the onset of darkness (20:00 h).

In the second series of experiments, 12 rats, pinealectomized 3 weeks before (obtained from Charles River Canada Inc.) and six sham-operated rats were used. They were adapted to the light-darkness conditions for 2 weeks before treatment. Six pinealectomized rats were injected intraperitoneally with melatonin (24 mg/kg) while six pinealectomized and sham-operated animals were similarly injected with vehicle. All the animals were injected at 16:00 h and were killed at 20:00 h (before the onset of darkness). they were anesthesized with Ketalar (100 mg/kg) and Xylosine (100 mg/kg) and then fixed for histological procedures as described below. Melatonin (RBI Research Biochemical International) was dissolved in 30% ethanol-saline.

In situ *hybridization*

The animals were fixed by intracardiac perfusion with 200 ml of 4% (wt/vol) PF in 0.1 M phosphate buffer (pH 7.4). The brains were then quickly frozen. Since it is now well established that, in the rat brain, the vast majority of GnRH-producing neuronal cell bodies are located in the preoptic area and anterior hypothalamus (Pelletier, 1980; Silverman *et al.*, 1987; Malik *et al.*, 1991), ten-μm thick coronal sections were serially cut through an area extending from the preoptic area to the anterior hypothalamus. The sections were then mounted onto gelatin- and poly-L-lysine-coated slides.

In situ hybridization was performed as described previously (Zoeller *et al.*, 1988; Toranzo *et al.*, 1989; Li & Pelletier, 1992). The probe chosen was a synthetic deoxyribonucleotide complementary to the GnRH coding region of the rat cDNA (bases 102–149) described by Adelman *et al.* (1986). This probe was synthesized in our laboratory using the Biosearch DNA synthesizer and purified on a 15% polyacrylamide/8M urea preparative sequencing gel. It was labelled on the 3′ end with [^{35}S]dATP (1000 Ci/mmole; Amersham Co., Oakville, Ontario, Canada) and terminal deoxynucleotidyl transferase (Boeringher Mannheim, Montreal, Canada) to a specific activity of about 5000 Ci/mmole.

The sections were prehybridized for 1 h in buffer A containing 50% (vol/vol) formamide, 5 × SSPE (1 × SSPE being 0.18 M NaCl, 10 mM NaH_2PO_4, pH 7.4, 1 mM EDTA), 0.1% (wt/vol) sodium dodecyl-sulfate, 0.1% (wt/vol) BSA, 0.1% (wt/vol) Ficoll, 0.1% (wt/vol) polyvinylo-pyrrolindone, 200 μg denatured salmon testis DNA/ml, 100 μg yeast tRNA/ml, and 20 μg poly(A)/ml.

Hybridization was carried out for 24 h at 42°C in buffer B (buffer A containing 4% (wt/vol) dextran sulfate) containing the labelled probe in a saturating concentration (10^7 c.p.m./section). After hybridization, sections were rinsed at room temperature for 2 h in 2 × SSC (1 × SSC being 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 2 h in 1 × SSC, 1 h in 0.5 × SSC and 1 h in 0.5 × SSC at 37°C followed by one rinse of 1 h in 0.5 × SSC at room temperature. Finally, the sections were dehydrated and coated with liquid photographic emulsion (Kodak NTB-2). After 20 days of exposure, the sections were processed and stained with hematoxylin-eosin.

To assess the specificity of the hybridization signal, consecutive sections were alternatively hybridized with the labelled oligonucleotide probe encoding GnRH and a labelled sense oligomer directed to the complementary DNA strand. As an additional control, sections from each group were treated with pancreatic RNaseA (20 μg/ml) (Boeringher

Mannheim, Montréal, Canada) for 30 min at room temperature before prehybridization.

Quantification of GnRH mRNA

Localization of neurons expressing GnRH mRNA as well as the count of grains per labelled neuron obtained after hybridization with the ^{35}S-labelled probe were performed in a random order by an observer unaware of the treatment received. A neuron was considered specifically labelled if the number of grains overlying the cell exceeded five times the background count. For each experimental group, the mean number of silver grains per cell was calculated from at least 250 ± 10 cells from six animals per group. Statistical significance was determined according to the multiple range test of Duncan-Kramer (Kramer, 1956).

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