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Melatonin increases nerve growth factor in mouse submandibular gland

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Abstract: The effect of melatonin administration on nerve growth factor (NGF) was studied in the submandibular glands of adult Swiss male mice. Melatonin injection, at 1 μg daily for 30 days, resulted in an increase in the NGF content as detected by immunohistochemistry. The submandibular gland weight and the area of the granular convoluted tubules, which contained NGF, were also increased significantly. These effects were not observed when the dose of melatonin was increased to 10 and 50 μg daily. None of the melatonin treatments used influenced the weights or histology of the testes or seminal vesicles of the mice. The results suggest that melatonin, at physiological concentrations, directly regulates NGF synthesis in the mouse submandibular gland.

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Introduction

Perez-Polo et al. [1978] reported that melatonin administration, blinding, or pinealectomy suppressed nerve growth factor (NGF) levels in the submandibular gland of Swiss mice. The findings seemed contradictory in the following sense. It is conceivable that melatonin administration and blinding, which might stimulate pineal activity and increase endogenous melatonin production [for review, see Reiter, 1991], would act in the same manner regarding NGF regulation. But the finding that pinealectomy, which leads to a decrease in melatonin levels, also suppressed NGF level is difficult to explain. It is possible that melatonin may have dual and opposite actions on NGF production, i.e., directly to stimulate NGF and indirectly to inhibit, by suppression of the hypothalamo-pituitary-gonadal axis. The indirect action is consistent with the finding that testosterone stimulates the synthesis of NGF in the mouse submandibular gland [Goldstein and Burdman, 1965; Ishii and Shooter, 1975; Walker et al., 1981]. Therefore, high doses of melatonin might suppress testosterone levels, which result in a reduced NGF synthesis. Alternately, melatonin could act only via the direct mode on the mouse submandibular gland and the dose-dependent results might be due to down-regulation mechanism. The doses of melatonin administered in the study by Perez-Polo et al. [1978] ranged from 0.05–2 mg, as a subcutaneous beeswax implant; these doses suppressed NGF concentration in the submandibular gland. In the present study, we administered lower doses of melatonin to the mouse and observed changes in NGF level in the submandibular gland, which suggests that melatonin directly stimulates rather than inhibits NGF production.

Materials and methods

Swiss male mice, aged 1 month, were divided into four groups of ten each. The groups were subcutaneously injected with 0.1 M, pH 7.4, phosphate-buffered saline (PBS) or melatonin (Sigma Chemical Co., St. Louis, MO) at doses of 1, 10, and 50 μg in 0.1 ml PBS, between 17.00–17.30 h daily for 30 days. The animals were housed in a 12:12 h light:dark room with light out at 18.00 h. At the end of the injection period, the mice were sacrificed and the submandibular salivary glands, testes, and seminal vesicles were individually isolated, weighed, fixed

(Bouin fixative, 24 h), processed through paraffin sectioning at 5 μm, stained with haematoxylin and eosin, and examined under a light microscopy.

The submandibular glands of all the mice were also stained immunohistochemically with NGF by using HISTOSTAIN™SP KIT (Zymed Laboratory, Inc., San Francisco, CA). The sections were deparaffinized and rehydrated through a graded series of ethanol. The activity of pseudoperoxidase was inhibited by immersing the slides in a 3% hydrogen peroxide solution for 10 min. After rinsing the sections for 5 min in PBS, a solution containing rabbit anti-NGF (Sigma Chemical Co., St. Louis, MO) was added and incubated at room temperature for 10 min. The sections were rinsed with PBS and incubated again with biotinylated secondary antibody at room temperature for 10 min. This was followed by rinsing and incubating with streptavidin-conjugated horse radish peroxidase (0.05 mg/ml in PBS) at room temperature for 10 min. After rinsing with PBS for 15 min, the chromogen 3-amino-9-ethyl-carbazole in hydrogen peroxide solution was added; this was followed by counterstaining with haematoxylin for 3 min. The sections were then rinsed, mounted, and examined under a light microscopy. Attention was paid on the granular convoluted tubules (GCT) of the gland, which contain NGF [Levi-Montalcini and Angeletti, 1968; Levi-Montalcini, 1982; Hazen-Martin and Simson, 1984]. The photographs of the NGF-containing granular convoluted tubules were outlined with Indian ink (Fig. 1); the areas of the GCT and the total section area were scanned and determined by a computerized software program Adobe Photoshop. The ratio of the GCT to the total section area was calculated. Five sections were randomly selected per animal; the ratio were calculated and averaged for each animal.

Numerical data were analyzed by ANOVA, followed by Newman-Kuels test.

Results

After 1 month of treatment, the body weights and the weights (both absolute and relative) of the testes and seminal vesicles did not differ significantly among the groups (data not shown). Histologically, the testes and seminal vesicles were also similar in all groups. The relative weights of the submandibular glands in melatonin group, however, were significantly greater than those of the controls ($P<0.05$, Fig. 2). The largest increase was seen in the group treated with 1 μg melatonin.

Immunohistochemical staining for NGF in the mouse submandibular salivary glands is shown in Figure 3. The peripheral part of the gland stained more intensely than the central portion, with NGF-reactive red granules distributed throughout the cytoplasm and sparsely in the nuclei (Fig. 3A). Some nuclei of the tubular cells located in the central

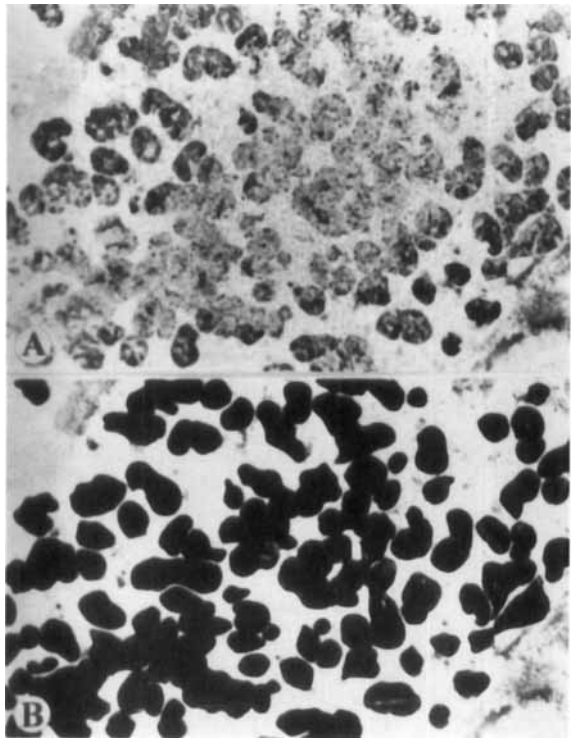

Fig. 1. The GCT of the mouse submandibular salivary gland staining immunohistochemically for NGF (**A**). The same section was blacken on the GCT area for area calculation (**B**). Original magnification, ×25.

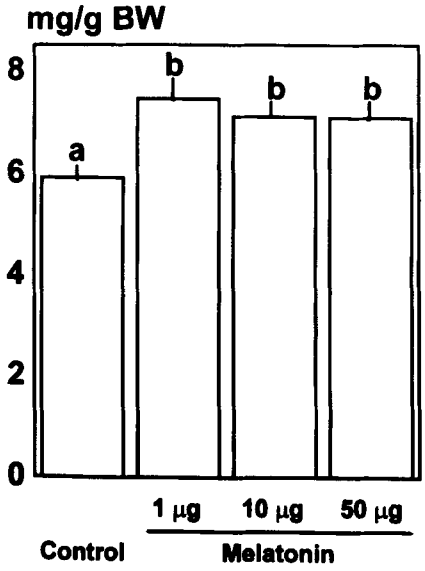

Fig. 2. Relative weights of the submandibular glands of the mouse, comparing between control and the melatonin groups. Different superscripts of the columns indicate the difference at statistically significant level ($P<0.05$).

portion stained red (Fig. 3B); this staining was non-specific as described by other investigators [Hazen-Martin and Simson, 1984]. Certain lobules of the GCT in the inner zone had intermediate levels of NGF in the cytoplasm (Fig. 3C). When comparing the NGF-immunohistochemistry of the GCT from different groups of mice, those that were treated with 1 μg melatonin revealed more granules in the cytoplasm than the other groups (Fig. 3E, compared to Fig. 3D, control; Fig. 3F, 10 μg melatonin; and Fig. 3G, 50 μg melatonin groups). The difference was more readily apparent in the central portion of the gland than the periphery; centrally, most of the cells contained scattered NGF granules with the features similar to those depict in Figure 3C. When the ratio of the GCT area and the total section area was calculated, it was found that the ratio of the 1 μg melatonin group was significantly higher than that of the control ($P<0.01$), and that of the 10 μg ($P<0.05$) and the 50 μg melatonin groups ($P<0.01$) (Fig. 4). A slight, non-significant, decrease of the ratio was observed in the 50 μg melatonin group.

Discussion

The increase in weight of the mouse submandibular gland induced with the 1 μg melatonin injection was accompanied by the increase in the relative area of GCT, as well as in its NGF content. Therefore, it is likely that the melatonin treatment stimulated NGF production in the GCT and increased the volume of GCT, which subsequently contributed to the increase in the gland weight. Questions raised at this point are how 1 μg melatonin treatment stimulated the production of NGF while the higher doses of melatonin did not have this effect.

The finding that the weights of the seminal vesicles and testes of the mice and histology of the two organs were normal suggests that melatonin, at all doses tested, had no significant effects on reproductive function. Therefore, the possibility that melatonin increased the NGF content in the GCT via the suppression of the hypothalamo-pituitary-gonadal axis is unlikely. Evidence from other studies also suggest that melatonin, even at milligram levels, does not suppress reproductive function in the laboratory mouse (*Mus musculus*) [Turek et al., 1976].

Since the higher doses (10 and 50 μg) of melatonin did not suppress mouse reproductive physiology, other mechanisms explaining their non-stimulatory or probably suppressive effect on the NGF content must be considered; receptor down-regulation is a possibility. It is possible that the stimulation of the receptors requires an optimum dose of melatonin, with higher doses down-regulating the receptor. When Perez-Polo et al. [1978] implanted beeswax pellets containing melatonin doses ranging from 0.05–2.0 mg, they reported the inhibitory, rather than the stimulatory, effects on the NGF production. In these studies, melatonin treatments also did not suppress testicular weights of the mouse, suggesting that the suppressive effect on the NGF production was not secondary to gonadal suppression by melatonin. Thus, their results on submandibular NGF could also be explained by the same mechanism, i.e., down-regulation of the melatonin receptor. An indirect evidence suggesting down-regulation of melatonin receptor in female hamsters has been reported; melatonin injections in late afternoon suppressed reproductive activi-

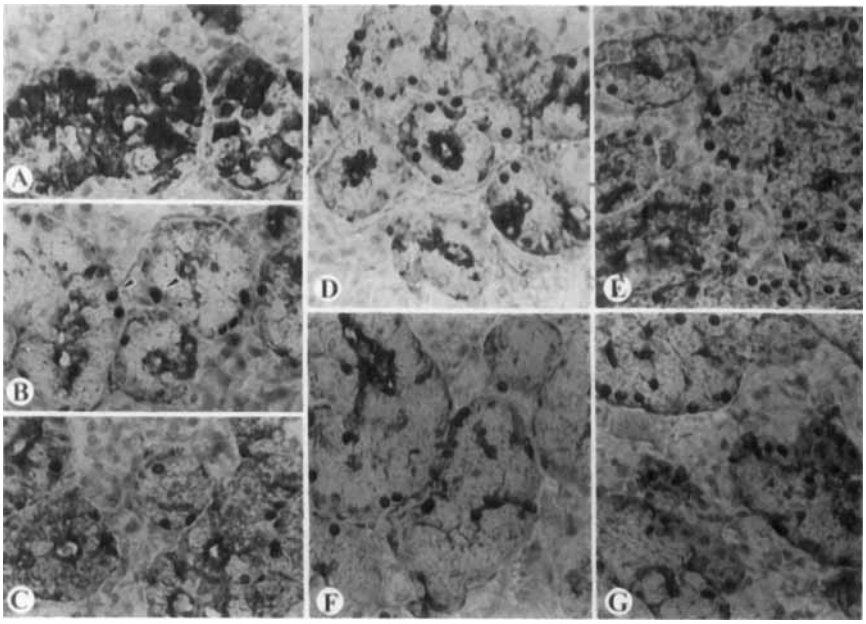

Fig. 3. Immunohistochemical staining of NGF in the mouse submandibular salivary glands. The GCT segment of the glands contained red granules in the cytoplasm which are more abundant in the outer zone (**A**) and either minimum (**B**) or intermediate amount of granule (C) in the inner zone. Some nuclei of the tubular cells stained red (B, arrow heads). The GCT of the 1 μg melatonin group (**E**) were visually more compact and contained more NGF granules than that of the control (**D**), the 10 μg melatonin (**F**), and the 50 mg melatonin (**G**) groups. Original magnification, ×100.

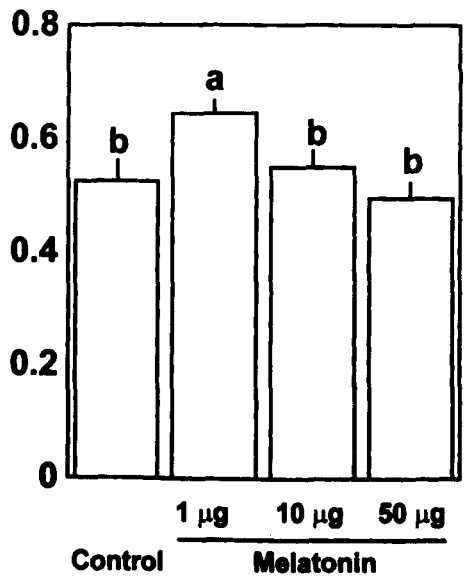

Fig. 4. Ratio of the areas of the GCT to the total section areas, comparing between control and the melatonin groups. Different superscripts of the columns indicate the difference at statistically significant level. $P<0.01$, comparing between the 1 μg melatonin and the control groups, and the 1 μg melatonin and the 50 μg melatonin groups. $P<0.05$, comparing between the 1 μg melatonin and the 10 μg melatonin groups.

ties and morning injection of the hormone prevented the effects [Chen et al., 1980]. Melatonin receptors have been demonstrated in brains, retina, pituitary gland, prostate gland, and tumor cells [Dubocovich, 1995; Reppert and Weaver, 1995; Gilad et al., 1996]. An indirect evidence suggesting the presence of melatonin receptors in the salivary glands is that high concentrations of melatonin were retained in palatal and submandibular salivary glands of the rat [Bubenik, 1980; Withyachumnarnkul et al., 1987]. Melatonin was also selectively accumulated in the nucleus of the gland cells [Withyachumnarnkul et al., 1987]. In golden hamster (*Mesocricetus auratus*), the effect of melatonin on NGF production could be via an indirect one as melatonin has a strong suppressive effects on its reproductive function in this species [Reiter, 1991]. When Uddin [1989] injected 25 μg of melatonin daily into adult male hamsters for 3 weeks, he found no change in the testicular weights or in the ultrastructure of the submandibular glands. If the injections were prolonged to 6 weeks, however, the testicular weights and the number of granules in the GCT were both decreased. Whether melatonin also has a direct stimulatory action on the hamster salivary gland needs further study; however, it might be difficult to distinguish the direct action from the indirect one as melatonin has an overwhelming influence on the reproductive function in this species.

At certain point in pineal research, there is some doubt on the presence of melatonin in the mouse. Without the presence of melatonin in the animals under studied, all the discussion above could be misleading. However, melatonin and its circadian rhythm were demonstrated in pineal glands and sera of certain strains of mice, especially the commonly used ones [Goto et al., 1989; Conti and Maestroni, 1996]. We therefore assume that the Swiss mouse used in this study had melatonin.

Physiological significance of melatonin in stimulating NGF awaits further studies. It is, however, interesting to note that both melatonin and NGF share a similar function in extending life of cells. NGF is a trophic factor for sensory neuron, sympathetic neurons, and basal forebrain neurons [Gotz and Schartl, 1994], whereas, melatonin acts as a scavenger to reduce hydroxyl radicals in cells [Tan et al., 1993]. Their concerted actions could be of significance in keeping normal life span of organisms.

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